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CELL CULTURE METHODS FOR THE SELECTION OF OSTEOBLAST-LIKE CELLS FROM THE PERIODONTAL LIGAMENT

A
THESIS

Presented to the Faculty of
The University of Texas Graduate School of Biomedical Sciences
at San Antonio
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of the Requirements
for the Degree of
MASTER OF SCIENCE

By
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San Antonio, Texas

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CELL CULTURE METHODS FOR THE SELECTION OF OSTEOBLAST-LIKE CELLS FROM THE PERIODONTAL LIGAMENT

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DEDICATION

I would like to dedicate this thesis to my wife Karen and my son Justin. They have given the ultimate sacrifice of time and patience which has greatly contributed to the success of this endeavor. Their continued love and support will never be forgotten. I would also like to dedicate this thesis to my parents. One never realizes the impact that two individuals can have on the successes encountered throughout life. My personal and educational values are a testament to the sacrifices they have made and the encouragement they have always provided.

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CELL CULTURE METHODS FOR THE SELECTION OF OSTEOBLAST-LIKE CELLS FROM THE PERIODONTAL LIGAMENT

Publication No. _______

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Periodontal regenerative therapy involves restoration of lost periodontal architecture through the coordinated efforts of fibroblasts, osteoblasts and cementoblasts. The periodontal ligament plays an important role in this process because it is thought to contribute necessary progenitor cells for regeneration. Numerous reports indicate phenotypic heterogeneity among cells of the periodontal ligament. In addition to populations of cells exhibiting a fibroblast

phenotype, cells with the capacity to form mineralized tissue have also been identified. A better understanding of these mineral-forming cells will contribute to the development of future regenerative therapies. The aim of this investigation was to use cell culture techniques to establish cell populations from human periodontal ligament enriched in the osteoblast-like phenotype. A second aim was to establish continuous periodontal ligament cell lines representative of both fibroblast-like and osteoblast-like cell types for future *in vitro* investigations.

Twenty-nine cell populations, established from explanted human periodontal ligament tissue, were expanded in three types of culture medium. In addition to standard Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, selection for an osteoblast-like phenotype also utilized low-calcium medium and Dulbecco's Modified Eagle's Medium containing 5% platelet-poor plasma. Following expansion, characterization indicated that three cell populations established in Dulbecco's Modified Eagle's Medium and one cell population established in low calcium medium were positive for mineralization in the presence of 2.5 mM β-glycerophosphate. These four cultures responded to $1,25(OH)_2$ Vitamin D_3 stimulation with increases in osteocalcin production greater than 2.75 ng/ml and significant increases in alkaline phosphatase activity. In addition, these cell populations responded to parathyroid hormone stimulation with a decrease in alkaline phosphatase activity and an increase in intracellular cyclic AMP production. While other cell populations established from periodontal ligament in this study exhibited some of these osteoblast-like characteristics, only mineralizing cultures displayed all evaluated characteristics. Analysis of the phenotypic characteristics expressed by all established cell populations indicated that selection for an osteoblast-like phenotype did not occur in the low

calcium or platelet poor plasma containing media evaluated.

Selection of the osteoblast-like phenotype was also attempted by evaluating cultures acquiring anchorage independent growth in a soft agar transformation assay. One clonal cell population, designated 14-4, was successfully expanded following colony formation in the soft agar. When characterized, this cell population did not mineralize in culture. Cell population 14-4 did exhibit an increase in alkaline phosphatase activity in response to $1,25(OH)_2$ Vitamin D_3 and an increase in intracellular cyclic AMP in response to parathyroid hormone. During expansion, this cell population underwent spontaneous transformation and has subsequently been passaged over 50 times indicating it is a stable cell line.

Two periodontal ligament derived cell populations designated 13-1 and 16-3 were transformed using a recombinant defective retrovirus, containing temperature sensitive SV-40 large T antigen cDNA and the neomycin resistant gene. Successful transformation was verified using immunohistochemistry to evaluate expression of the large T antigen. Prior to transformation, both cell populations expressed a phenotype consistent with cells capable of mineralization. Following transformation, cell population 13-1 retained characteristics consistent with the osteoblast-like phenotype, while cell population 16-3 no longer expressed this phenotype and was characterized as fibroblast-like. Cell population 13-1 continued to express the osteoblast-like phenotype when cultured at the non-permissive temperature of 37°C when expression of the large T antigen was turned off. Further study using a previously transformed human periodontal ligament cell line designated HPDL, evaluated cell proliferation at both the permissive temperature of 33°C and the non-permissive temperature of 37°C. Cell proliferation was decreased at the higher temperature when compared to cells still expressing the

transformation gene. Immunohistochemistry verified that the large T antigen was not expressed at 37°C. These results indicate that cell populations placed at the non-permissive temperature can be studied under conditions when they resemble non-transformed cells in primary culture.

Although the alternative culture methods did not result in selection of cell populations from periodontal ligament enriched in the osteoblast-like phenotype, the results from this study provide further evidence for heterogeneity with respect to cell types present in the periodontal ligament. In addition, three newly transformed cell lines have been established and are available for future study of the regenerative potential of cells resident in the periodontal ligament.

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I. INTRODUCTION AND LITERATURE REVIEW

A. Periodontal Regeneration

The goal of periodontal therapy is to stop disease progression and re-establish lost periodontal architecture through regeneration of a functional attachment apparatus composed of new bone, cementum, and periodontal ligament (PDL). Periodontal therapy can halt the destructive nature of periodontitis but the regeneration of lost tissue is difficult and unpredictable. Regeneration is the process by which architecture and function are completely renewed (Hancock, 1989). For this to occur following periodontal therapy, there must be formation of a new periodontal attachment apparatus which requires the coordinated efforts of fibroblasts, osteoblasts, and cementoblasts (Melcher, 1976).

Oral tissues, including epithelium, gingival connective tissue, PDL, and alveolar bone, are found to participate in the healing process following periodontal surgery. When gingival flaps are reapposed, lost tissue attachment to the root surface heals by a long junctional epithelial attachment (Listgarten and Rosenberg, 1979; Stahl *et al.*, 1982). This occurs because oral epithelium migrates to the wound preferentially over other tissues and forms a new attachment (Braga and Squier, 1976). In most clinical situations flap closure is followed by formation of a long junctional epithelial attachment between the root surface and the soft tissue flap, with epithelial cells consistently located at or close to their presurgical level. While a long junctional epithelial attachment is considered an adequate form of repair (Magnusson *et al.*, 1983; Beaumont *et al.*, 1984), regeneration is not accomplished. In the absence of regeneration healing by repair occurs. Studies have shown that gingival connective tissue and alveolar bone may cause

resorption and ankylosis of the root surface when healing occurs by repair. (Line *et al.*, 1974; Nyman *et al.*, 1980; Karring *et al.*, 1980; Aukhil *et al.*, 1986). In order to achieve regeneration rather than healing by repair, epithelium and gingival connective tissue must be excluded from the wound site, thereby allowing repopulation of the site with PDL and alveolar bone cells (Melcher, 1976; Nyman *et al.*, 1982). This concept is the basis for guided tissue regeneration (GTR).

B. Progenitor Cells

In order to restore lost structure and function of the periodontium following disease or surgery, there must be a coordinated effort by progenitor cells to regenerate lost bone, cementum, and functionally oriented PDL fibers. Successful GTR procedures indicate that necessary progenitor cells are present, and that proliferation, migration, differentiation, and synthesis of extracellular matrix components by their progeny are necessary for formation of these tissues. PDL cells play an important role in the regenerative process (Melcher, 1976; Egelberg, 1987), and it is thought that the PDL contains the progenitor cells necessary for restoration of structure and function of lost tissue (Melcher, 1976; Gould, 1983). It is not known whether a single progenitor resides in the PDL that gives rise to cells which differentiate into fibroblasts, osteoblasts, and cementoblasts or whether there are separate progenitors for each phenotype. In normally functioning periodontal tissues, cell turnover of PDL fibroblasts comprises a steady state renewal system whereby the generation and proliferation of new cells is balanced by apoptotic cell death. PDL cell progenitors can generate large numbers of fibroblastic cells and more limited numbers of osteogenic and cementogenic cells (McCulloch, 1995; Lekic and McCulloch, 1996). It has been reported that under physiologic conditions (McCulloch and Melcher, 1983) or following wounding in the PDL (Gould *et al.*, 1977), dividing cells with many characteristics of progenitor

cells are located paravascularly. Using nuclear morphometry, it was also shown that paravascular fibroblast-like cells may be progenitors of osteoblasts (Roberts *et al.*, 1987). Also, it has been suggested that osteoblast and cementoblast progenitor cells may originate from the endosteal spaces of bone and migrate into the PDL (Melcher *et al.*, 1986; McCulloch *et al.*, 1987). However, more recent studies do not report an increase in the number of paravascular or endosteal cells during new bone formation. Studies examining extraction socket healing (Lin *et al.*, 1994) and regeneration of the furcation in the beagle dog (Herr *et al.*, 1995), suggested that osteoblast precursors were supplied locally, and that paravascular and endosteal cells played a minor role in new osseous tissue formation. While these studies report conflicting origins for progenitor cell populations involved in healing, their results are consistent with the theory that the PDL contains a heterogenous population of cells capable of regenerating lost periodontal tissue. Thus, the PDL is extremely important during regeneration of a functional attachment apparatus.

C. Periodontal Ligament

The periodontal ligament is the soft connective tissue interposed between the roots of the teeth and the inner wall of the alveolar socket. Healthy periodontal ligament contains a number of different cell populations including fibroblasts, osteoblasts, cementoblasts, endothelial cells, epithelial cell rests of Malassez, and cells associated with the sensory system. The fibroblast is the predominant cell type which occupies between 20-35% of the PDL space depending on the animal/human model, and the type of tooth evaluated (Beertsen *et al.*, 1997). The major fibers of the PDL are composed of collagen bundles consisting of cross-banded fibrils. These collagen fiber bundles are mainly composed of collagens I and III, although collagens V, VI and XII have also been reported. Temporal and spatial expression of collagens I and XII in the remodeling

periodontal ligament during experimental tooth movement suggests that collagen XII is closely associated with regeneration of PDL function (Karimbux and Nishimura, 1995). Besides collagen, oxytalan fibers, composed of a high molecular weight glycoprotein resembling pre-elastic fibers, have also been reported in the PDL (Fullmer *et al.*, 1974; Shuttleworth *et al.*, 1983).

The PDL is characterized by rapid turnover and a high remodeling rate involving rapid synthesis and breakdown of matrix components, most notably the abundant collagen fibers. This remodeling is necessary for functional adaptation of the PDL in response to positional changes of the teeth. Remodeling of the PDL apparently occurs by different processes depending on the inflammatory state of the tissue (Beertsen *et al.*, 1997). Under steady state conditions, PDL collagen turnover is accomplished through the process of phagocytosis by resident fibroblasts, with degradation occurring in lysosomal structures utilizing cysteine proteinases (Everts *et al.*, 1996). However, during inflammation, collagen degradation occurs extracellularly through the action of collagenase, a member of the metalloproteinase family (Everts *et al.*, 1996). Whether during a state of health, inflammation or during mechanical tooth movement, the process of remodeling is paramount in maintaining a functional PDL.

Restoration of a functional periodontal attachment apparatus not only requires regeneration of the soft tissue elements by fibroblasts, but also regeneration of the mineralized components through the coordinated activity of osteoblasts and cementoblasts (Melcher, 1976). Thus, while fibroblast cell populations help maintain a steady state in the PDL during health, additional cell phenotypes are required during wound healing situations for the restoration of hard tissue lost to disease.

D. Phenotypes Found in the Periodontal Ligament

1. Fibroblasts. The expression of different phenotypic markers during *in vitro* studies of PDL explants has provided evidence for the cellular heterogeneity of this tissue. PDL fibroblasts have been well studied and differences both in growth characteristics and secretory products have been reported. Differences *in vitro* between collagen and non-collagen protein synthesis (Somerman *et al.*, 1988) and glycosaminoglycan synthesis (Mariotti and Cochran, 1990) have been reported between PDL and gingival fibroblasts and these functional differences evidence one phenotypic expression found in the PDL. Cloning procedures have revealed that PDL fibroblasts themselves represent heterogenous subpopulations with distinct functional characteristics based on extracellular matrix molecule expression. PDL clonal populations expressed differing amounts of collagen I and III as well as fibronectin when evaluated *in vitro* by immunocytochemistry (Hou and Yaeger, 1993). Such variances in phenotypes during characterization of PDL fibroblast subpopulations may represent cells at selected stages of differentiation, and explain the multiple functional characteristics reported for fibroblasts.

2. Osteoblasts Cells from the PDL have also been reported to express osteoblast-like characteristics *in vitro*. Production of predominantly type I collagen and osteonectin by porcine PDL cells was initially reported (Wasi *et al.*, 1984). High endogenous alkaline phosphatase activity in PDL cells (Somerman *et al.*, 1988; Nojima *et al.*, 1990; Ogata *et al.*, 1995; Goseki *et al.*, 1995; Giannopoulou and Cimasoni, 1996; Carnes *et al.*, 1997; Liu *et al.*, 1997) that is increased following stimulation by $1,25(OH)_2D_3$ (Kawase *et al.*, 1988; Piche *et al.*, 1989a; Carnes *et al.*, 1997) and decreased following exposure to parathyroid hormone (PTH) (Piche *et al.*, 1989a) demonstrates the presence of a marker enzyme normally associated with bone cells. PTH-induced increases in cyclic adenosine monophosphate (cAMP), another hormonal response

characteristic of osteoblasts has also been reported for PDL cell populations (Piche *et al.*, 1989a; Nojima *et al.*, 1990). Increased cyclic-AMP in response to PTH has also been noted in PDL cell cultures treated with dexamethasone (Nohutcu *et al.*, 1995). In this study, the potent synthetic glucocorticoid, dexamethasone, was reported to induce osteoblastic cell differentiation. Evidence for the presence of cells in the PDL with a hard tissue phenotype was further demonstrated by synthesis of osteocalcin (bone gla protein) in response to stimulation with $1,25(OH)_2D_3$ (Nojima *et al.*, 1990; Carnes *et al.*, 1997). However, not all *in vitro* assays have produced the same results. Some studies report an inability to demonstrate a PTH-induced cAMP response (Somerman *et al.*, 1990; Ogata *et al.*, 1995; Nohutcu *et al.*, 1995) while others fail to observe an osteocalcin release in certain cultures (Gosecki *et al.*, 1995; Ogata *et al.*, 1995; Nohutcu *et al.*, 1997; Carnes *et al.*, 1997). These conflicting results may be explained by the heterogenous nature of primary cell cultures obtained from the PDL. Mineralization is the hallmark of the functional phenotype of the osteoblast, and several recent studies report mineralization by PDL cells *in vitro* (Arceo *et al.*, 1991; Cho *et al.*, 1992; Mukai *et al.*, 1993; Nohutcu *et al.*, 1997; Carnes *et al.*, 1997). Furthermore, the potential for PDL cell cultures to mineralize is increased by the addition of dexamethasone. Mineralization nodules differed in appearance in these studies, and the difference was again attributed to the heterogeneity of the cell populations present in cultures, as well as whether or not osteoprogenitor cells were present in culture (Mukai *et al.*, 1993; Liu *et al.*, 1997).

3. Cementoblasts Another tissue type crucial for periodontal regeneration is cementum. Cell markers for cementoblasts have only recently become available. As a result, early investigators relied on morphological evidence as criteria for cementogenesis. It was shown by

co-culture *in vitro* with non-demineralized and partly demineralized root slices followed by reimplantation *in vivo* that cementum-like tissues were synthesized by fetal rat calvarial cells (Melcher *et al.*, 1986). Additional support for bone as the source of origin of cementoblasts resulted from *in vivo* studies using cultured alveolar bone cells from minipigs. When these cells were cultured on dental roots and reimplanted cementum-like tissue was synthesized on surfaces of the roots (Lang *et al.*, 1995). By contrast, PDL cells have been shown to form mineralized nodules *in vitro* which resemble acellular cementum (Cho *et al.*, 1992). A subsequent *in vitro* study also reported an acellular cementum-like material formed along dentin slices incubated with PDL explants high in alkaline phosphatase activity (Groeneveld *et al.*, 1994). An *in vivo* study by Aukhil *et al.* (1986) also reported that progenitor cells repopulating the wound site form cementum when they come in contact with root dentin. However, until an unequivocal cell marker for cementoblasts is identified, progenitors of these cells and their location, migration, differentiation and synthesis of cementum can only be postulated.

E. Phenotypic Markers of the Osteoblast-Like Phenotype

From the previous discussion it can be concluded that the PDL contains a heterogenous population of cells. Mass populations of cells grown from tissue explants of PDL are heterogenous in a number of morphological, growth and biochemical characteristics and some of these traits have provided evidence for the existence of an osteoblast-like phenotype.

1. Alkaline Phosphatase. Alkaline phosphatase occurs primarily as a high molecular weight (140,000 dalton) glycoprotein enzyme bound to cell membranes by a glycan phosphatidylinositol moiety (Moss, 1997). There appears to be a temporal sequence of gene expression associated with the development of an extracellular matrix during the process of

mineralization. Following the down-regulation of proliferation in cell cultures, there is an increased expression of alkaline phosphatase. This increase, prior to the initiation of osteoblast mineralization, suggests that alkaline phosphatase may be involved in preparation of the extracellular matrix for the ordered deposition of mineral (Lian and Stein, 1992). This increased expression following decreased proliferative activity has been shown in cell culture using a cell line, ROS 17/2.8 cells, which possesses characteristics of mature osteoblasts (Majeska *et al.*, 1985), as well as in culture of primary fetal rat calvarial cells (Lian and Stein, 1992, Harris *et al.*, 1994). Alkaline phosphatase may promote mineralization through one or more of the following mechanisms: (1) hydrolysis of ester phosphate yielding a local enrichment of orthophosphate for incorporation into nascent $CaPO_4$ mineral; (2) hydrolysis of local pyrophosphate to yield two molecules of orthophosphate used in the formation of nascent $CaPO_4$; and (3) alkaline phosphatase phosphotransferase activity which may translocate phosphate groups from the extracellular fluid to nearby substrates creating phosphate-enriched regions of the matrix vesicle membrane that may serve as a nidus for the nucleation of hydroxyapatite (Anderson, 1995). Alkaline phosphatase is a characteristic enzyme of osteoblasts, but isoenzymes exist in the kidney, liver, placenta and intestine (Rodan and Rodan, 1984). Using human PDL cell cultures, it has been shown that the alkaline phosphatase expressed is of the nonspecific type isolated from osteoblastic cells (Goseki *et al.*, 1995).

2. Osteocalcin (Bone Gla Protein). Osteocalcin is a vitamin K-dependent calcium binding protein with a molecular weight of 5,800 daltons. It is characterized by three γ-carboxyglutamic acid residues (Gla) that bind tightly to hydroxyapatite and is one of the most abundant noncollagenous proteins accumulated in bone tissue (Hauschka *et al.*, 1989). The

appearance of osteocalcin late in the osteoblast developmental sequence makes it a marker of the mature osteoblast (Lian and Stein, 1995). Osteocalcin has been shown to contribute to regulation of the mineral phase of bone as a potential inhibitor of mineral nucleation *in vitro* (Boskey, 1985) and as a bone matrix signal that is involved in osteoclast recruitment and differentiation *in vivo* (Glowacki and Lian, 1987). Osteocalcin is also present in odontoblast cell cultures and thus may be a marker of cells capable of synthesizing and secreting an extracellular matrix which mineralizes (MacDougall *et al.*, 1998)

3. <u>Cyclic AMP</u>. Cyclic 3′, 5′-adenosine monophosphate (cAMP) is an intracellular second messenger that results from the transformation of ATP when catalyzed by the enzyme adenylate cyclase. The stimulation of cAMP production by human parathyroid hormone (PTH) is characteristic of the osteoblast phenotype (Auf'mkolk *et al*, 1985). There appears to be a negative-feedback loop between cAMP and Ca^{++} that, under certain conditions, results in stable oscillations of the concentrations of both intracellular messengers.

4. <u>Mineralization</u>. Mineralization is the functional phenotypic marker of the mature osteoblast. Seventy to 80% of the total mass of bone is composed of mineral made up of hydroxyapatite ($Ca_6(OH)_{10}PO_4$). Organic constituents make up the remainder of bone and consists mainly of type I collagen which accounts for 80-90% of the extracellular matrix protein (Boskey, 1989). Collagen is the major synthetic product of osteoblast cultures *in vitro*. Mineral deposition in bone, dentin and cementum occurs in association with the collagen fibrils. Formation of the collagenous matrix contributes to the shutdown of proliferation that is needed for the induction of certain genes which contribute to the developmental sequence of osteoblast differentiation and subsequent mineralization (Lian and Stein, 1992). Mineralization of bone cell

cultures *in vitro* requires certain levels of calcium, phosphorus and ascorbic acid, and is modulated by $1,25(OH)_2D_3$ and dexamethasone. Mineralization in culture by osteoblasts has been shown to recapitulate events associated with mineralization *in vivo* and result in mineral-matrix associations similar to bone mineral (Bhargava *et al.*, 1988; Harris *et al.*, 1994). Therefore, the ability of a cell to mineralize in culture is a hallmark for the hard tissue phenotype.

F. Rationale for Cell Culture Studies.

It is evident from the previous discussion that the PDL contains a heterogenous population of cells able to express different phenotypes necessary for periodontal regeneration. These cells with multiple phenotypes interact in the regeneration process and may be regulated by many factors; such as locally active paracrine and autocrine polypeptide growth factors, extracellular matrix molecules, inflammatory mediators, hormones, and prostaglandins. *In vivo* experiments have shown that locally introduced cytokines and growth factors can modulate the regenerative potential of these cell populations through their mitogenic and chemoattractant properties (Lynch *et al.*, 1991; Rutherford *et al.*, 1992; Sigurdsson *et al.*, 1995; Cho *et al.*, 1995; King *et al.*, 1997; Howell *et al.*, 1997). In order to successfully and predictably apply these locally active factors to the regenerative process, *in vitro* experiments are necessary to help define the cellular relationships that occur *in vivo*. Phenotypic expression of fibroblast and osteoblast-like cell populations has been found in the PDL as already described. Information on the osteoblast-like properties of cells from the PDL is of particular interest to clinical investigators concerned with reestablishing lost bone and cementum during periodontal regeneration.

By using PDL explants *in vitro*, it is feasible to identify characteristics associated with different PDL cell populations and to view the mechanisms regulating their activity (Brunette *et*

al., 1976). However, PDL explants contain a heterogenous population of cells that can create inconsistencies when characterizing for osteoblast-like properties. Phenotypic expression of osteoblast-like cells may be due to both the paracrine factors produced by the surrounding tissue and the relative level of differentiation of the cells themselves (Pitaru *et al.*, 1994). Furthermore, the secretory phenotype can vary with respect to the cell division cycle (Ko *et al*,. 1981; Mariotti and Cochran, 1990). Bone research has shown that heterogeneity exists among cells of the osteoblastic lineage (Bellows and Aubin, 1989; Zhang *et al.*, 1991). This may be due either to the osteoprogenitor cell assuming different stages of differentiation and /or to a subspecialization of an already terminally differentiated osteoblast (Guenther *et al.*, 1989). This concept supports the studies of Lian and Stein (1992) where *in vitro* maturation of the osteoblast phenotype displays a temporal sequence of gene expression. The peak levels of expressed genes reflect a developmental sequence of bone cell differentiation characterized by three principal periods: proliferation, extracellular matrix maturation and mineralization. For these reasons experimental studies are difficult to interpret because of uncertainty as to which cell types respond to exogenously added stimuli such as different hormones or cytokines (Guenther *et al.*, 1989). Clonal cell populations are able to overcome the disadvantages seen in heterogenous tissue explants by providing homogeneous cell populations (Guenther *et al.*, 1989; Hou and Yaeger, 1993). Different clonal populations of PDL derived cells permit observation of the effects of differently applied local factors to osteoblast-like cells and, therefore, may provide insight into the regulatory mechanisms of periodontal regeneration so that *in vivo* application of these same factors may become more predictable.

From the previous discussion it is evident that osteoblast-like cells are present in the PDL

and are an important phenotype for periodontal regeneration. Therefore, *in vitro* study of these cells is necessary to elucidate the phenotypical characteristics present. Because of the heterogenous population of cells found in the PDL, inconsistencies can develop during characterization. It would be advantageous to study a homogenous population of cells that could be arrived at through clonal techniques. Limiting dilution cloning techniques are not efficient (Martin, 1994) and it would, therefore, be beneficial to select out the desired cells (osteoblast-like) prior to cloning. In this way, selection of osteoblast-like cells *in vitro* from a heterogenous PDL would allow future studies using a more efficient clonal technique to observe homogenous PDL cell populations at different stages of development along an osteogenic pathway.

G. Selection of an Osteoblast-Like Phenotype.

1. Selection Medium. Selection of osteoblast-like cells from bone tissue has been reported by using selection medium during *in vitro* culturing of explants. Using low-calcium culture medium, Robey and Termine (1985) were able to establish osteoblastic cell populations *in vitro* from iliac crest and long bone tissue explants confirmed by characterization for osteoblast cell markers. These cell markers included alkaline phosphatase production, increased cAMP in response to PTH, mineralization, production of type I collagen without type III production, and synthesis of osteonectin and bone proteoglycan. Since PDL cell cultures are heterogenous and contain cells with an osteoblast-like phenotype, culture of PDL cells in low-calcium medium may select for this phenotype. Similarly, osteoblasts have been shown to proliferate in platelet-poor plasma while fibroblasts do not. This is apparently due to the fact that osteoblasts not only respond to PDGF but synthesize this growth factor as well. This potential autocrine regulatory pathway is absent in fibroblasts (Zhang *et al.*, 1991; Piche *et al.*, 1989b). Piche *et al.*, (1989a)

reported that culture of PDL cells in medium containing platelet-poor plasma results in the selective proliferation of cells with an osteoblast-like phenotype compared to fibroblast-like cells, suggesting that this mode of culture may be an efficient mechanism for selecting this phenotype.

2. Clonogenic Growth in Semisolid Medium. Selection of osteoblasts has been accomplished using soft agar cloning techniques and TGF-β to induce anchorage-independent growth (Guenther *et al.*, 1989). It has been shown that bone cells can be induced by TGF-β to assume reversibly a transformed phenotype (DeLarco *et al.*, 1978). This occurs when anchorage-dependent cells acquire the ability to grow in semisolid medium. The resultant cell populations present in large colonies derived from a single progenitor presented with distinct osteoblast phenotypes (Guenther *et al.*, 1989). Using this technique, Guenther *et al.*, (1989) were able to grow and expand clonal cell populations from postnatal rat calvaria in soft agar. Because of the inherent heterogeneity found in rat calvarial tissue, explantation and characterization can yield different phenotypes. However, following growth in the soft agar assay, expansion and characterization revealed 48 cell clones with markers for the osteoblast phenotype. While most of the clonal populations possessed all markers evaluated for the osteoblast phenotype, some clones only expressed a few markers and it was suggested that these cell populations represented stages of differentiation along the osteogenic pathway. It is hypothesized that, using similar methodology, clonogenic growth of PDL cells in semisolid medium may selectively yield cell populations with osteoblast-like properties.

H. Immortalization.

The ability to select and characterize cells derived from PDL tissue as osteoblast-like provides an important tool to increase our understanding of the role of these cells in regeneration

of the hard tissue components of the periodontal attachment apparatus. The ability to establish stable PDL cell lines, which express tissue-specific gene products using the technique of immortalization, would greatly facilitate such studies. Currently, problems exist with techniques of isolating and expanding PDL tissue *in vitro*. The major problem is that PDL cell populations undergo phenotypic changes and senescence during long term maintenance in culture following repeated cycles of proliferation and replating (Adams *et al.*, 1993). As a result, prolonged study of cells with specific phenotypes relies on the repeated isolation and characterization of cells from fresh tissue. In addition, long term studies are difficult not only because they rely on repeated isolation of PDL cells from different batches of fresh tissue over time, but also because the resultant cell cultures are heterogenous with respect to cell phenotype. This often makes interpretation of results difficult. Thus, immortalization of PDL cells of a selected phenotype would enhance investigation into the functional and regulatory mechanisms of osteoblast-like cells and their role in periodontal regeneration.

Immortalization of cells permits maintenance of cell populations *in vitro* over many passages without limitations on life span. Immortalization may be caused by spontaneous mutations, exposure to chemical carcinogens or by viral infection. To date, only one reference of immortalized human PDL cells has been reported (Hoang *et al.*, 1997a). In this study, viral infection by simian virus 40 (SV-40) encoding for the large tumor antigen (T antigen) allowed for cellular transformation. Resultant population doublings in excess of 30 times were reported. Such an increase in the life span of cells in culture is one characteristic of immortalized cells. Regulation of growth control, however, may uncouple the relationship between proliferation and differentiation found in normal diploid cells (Lian and Stein, 1992). This pertains to osteoblast-

like cells where the normal ordered expression of genes encoding for the osteoblast developmental sequence may lose the observed transition points as the cell progress from the proliferative stage into the differentiation stage where development of an extracellular matrix which ultimately becomes mineralized occurs. Thus, while immortalized cell populations overcome limitations on long-term maintenance *in vitro*, phenotypic characteristics may be altered with respect to primary cell cultures that are not transformed. In order to overcome this potential problem of immortalization, a unique immortalization protocol has been developed. SV-40 large T antigen oncogene has been constructed to encode for a thermolabile large T antigen gene (Jat and Sharp, 1989). This gene allows cells to proliferate continuously at the permissive temperature of 33^{0}C without alteration of phenotypic expression. When placed at an elevated temperature (37-39^{0}C) the large T antigen is no longer expressed while phenotypic expression continues unaltered. Thus, this construct permits expression of normal cellular characteristics in a population of previously transformed cells, in which the factor responsible for transformation has been turned off. In short, this immortalization technique permits maintenance of cells in culture for many population doublings, yet permits studies to be performed in the absence of expression of the transforming factor when the cells resemble primary cultures. Application of this technique to PDL cells selected and characterized by known criteria will permit long term studies using defined cell lines of the role of PDL cells in regeneration of the periodontal apparatus.

II. METHODS

A. Periodontal Ligament Explants

1. Collection of Samples. All human PDL tissue samples were obtained at the University of Texas Health Science Center under Institutional Review Board protocol # 934-1904-205. Periodontal ligament tissue was obtained from teeth extracted during treatment of patients in the graduate oral surgery and hospital dentistry clinics. A total of 136 teeth were obtained from 47 individuals (27 female; 20 male) whose age ranged from 15-60 years. The majority of teeth were healthy, erupted third molars or premolars. In some cases, caries and attachment loss were noted. Extracted teeth were taken directly from the surgical site and immediately placed in 50 ml tissue culture tubes (Falcon) containing 30 ml of a sterile solution of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) and an antibiotic solution (1% PSF) (Gibco) composed of 100 units/ml of penicillin, 100 μg/ml of streptomycin and 2.5 μg/ml of amphotericin B. Culture tubes with tissue were taken directly to the laboratory for explanting. All subsequent steps were accomplished in a laminar flow hood using sterile technique.

2. Explant of Periodontal Ligament Tissue. Periodontal ligament (PDL) cells were explanted and cultured using techniques modified after Piche *et al.* (1989a) and Freshney (1992). Using a sterile Younger-Good curette, tissue was removed atraumatically, trying to avoid cementum removal, from the middle third of each root surface and placed into a plastic petri dish (Corning) containing a sterile solution of DMEM and 5% PSF. If more than one tooth was extracted from the same patient, periodontal ligament tissue was pooled in order to optimize the amount of available tissue. Tissue samples remained in this solution for approximately 10

minutes. The tissue was then removed, sectioned into approximately 1 mm^3 pieces, then radially arranged in a clock-face manner along the bottom of the wells of 6-well culture plates (Corning). Each plated well contained between 8-12 pieces of periodontal ligament tissue. To facilitate attachment to the bottom of the culture plate, the plate was allowed to dry in an incubator at 37^0C for five minutes. After attachment, 3 ml of culture medium (described below) was added to each well. The explanted tissue was then incubated under standard conditions in a humidified environment at 37^0C in an atmosphere of 95% air and 5% CO_2. Time from tooth extraction to placement of tissue into the incubator varied from 20 minutes to 2 hours. Medium was changed weekly in these primary cultures until cell migration from the explant onto the surface of the well was observed. At the time cell migration from the explanted tissue was appreciable, frequency of medium changes was increased to every 3-4 days. Upon reaching a confluent monolayer, cells were transferred from the well. The existing culture medium was evacuated and the cells were washed for one minute with Hanks Balanced Salt Solution (HBSS) (Gibco). Cultures were then trypsinized using 0.05% trypsin-EDTA solution (Gibco) and transferred to a 25 cm^2 flask (Corning) and designated passage one. After reaching confluence, cells were similarly passaged at a one to three ratio until four confluent 75 cm^2 flasks (Corning) were obtained (typically fourth through six passage). Final passage cells were trypsinized and pooled for plating as required by the design of the characterization experiments. Cells were not used past the ninth passage to avoid cells undergoing differentiation or senescence (Adams *et al.*, 1993).

3. Handling of the Cell Populations. If explanted PDL tissue did not exhibit initial cellular outgrowth after 4 weeks in culture, it was then discarded. Cell cultures which ceased to proliferate or that became contaminated were similarly discarded. Following final passage,

remaining cells not used in characterization experiments were frozen (1-2 million cells in a 1 ml cryotube) using 1 ml of 10% DMSO (Sigma) and 90% FBS. Cells were placed in a styrofoam container and maintained at -20^{0}C for 24 hours, -80^{0}C for another 24 hours, and finally placed into liquid nitrogen for long-term storage. Rapid thawing (37^{0}C water bath) was used when bringing cells out of the frozen state to initiate new cultures.

At the start of characterization, the cells were plated at a specific density which varied for each protocol (detailed below). Once cell growth reached confluence before final passage, the cells were washed once with HBSS and trypsinized to a 50 ml culture tube. Cell number was determined using a Coulter Counter (model Z_F, Coulter Electronics) by diluting a fraction of the cell suspension in 10 ml of Isoton (Stephens Scientific). Three cell counts were taken, averaged and the cell number was calculated for the total cell suspension. An appropriate volume of cell suspension was added to the culture medium used in each characterization experiment. Culture medium in which the primary cultures were established was used during cell characterization experiments for that respective cell line unless otherwise stated. Cell morphology of all cultures was observed and photographed using an Olympus (Ck2) phase contrast microscope.

B. Culture Medium.

Three different types of culture media (test medium) were used to facilitate cell growth from explanted periodontal ligament tissue.

1. DMEM. Standard culture medium for explant and culture of PDL cells was DMEM supplemented with 10% fetal bovine serum (FBS)(Summit, Fort Collins, CO) and 1% PSF.

2. Low-Calcium Medium. A low calcium culture medium (Robey and Termine, 1985) was also used for explant and culture of PDL tissue in an attempt to establish cultures enriched in

osteoblast-like cells. A 1:1 mixture of DMEM and F12 was prepared calcium free by Gibco, Grand Island New York, and supplemented with 10% FBS and 1% PSF. While the serum free DMEM/F12 medium lacks Ca^{++}, it was estimated that calcium levels were in the rage of 0.2 mM due to the Ca^{++} present in FBS (Robey and Termine, 1985). It has also been shown that PDL cell attachment and growth is possible in low calcium medium (Lengheden, 1994). To allow for cell attachment, cell populations were passaged using DMEM then switched to low-calcium medium within 24 hours.

3. Platelet-Poor Plasma Medium. In an attempt to obtain cultures enriched in osteoblast-like cells, DMEM supplemented with 5% platelet-poor plasma (PPP)(BTI, Stoughton, MA) and 1% PSF was also used for explant and culture of PDL tissues (Piche *et al.*, 1989a).

C. Cell Characterization.

1. Alkaline Phosphatase Biochemical Assay. Cells were plated at a density of 25,000 cells/cm^2 in 72 wells of a 96 well cell culture plate and grown to confluence. Culture medium was removed and the cells rinsed with 200 µl of HBSS. Cells were stimulated in wells with 150 µl of serum free test medium containing 0.1% bovine serum albumin (BSA) (Miles Scientific) and 1% PSF alone or supplemented with concentrations of 2.35×10^{-8} $1,25(OH)_2D_3$ (VitD) or 100 ng/ml parathyroid hormone (PTH) (Bachem, Torrance, CA). Six replicates were evaluated for each concentration. Following incubation at 37^0C for 48 hours the medium was removed and the cells rinsed three times with phosphate buffered saline (PBS). Sixty µl of 1% triton-X-100 (Sigma) was added and the wells incubated at 37^0C for 20 minutes to solubilize cell membranes.

Alkaline phosphatase activity was assayed by adding 50 µl of assay buffer containing a 1:1:1 ratio of 1.5 M 2-amino-2 methyl-1-propanol (EM Science), 20 mM para-nitrophenol

phosphate (Sigma) and 10 mM $MgCl_2$ to 50 µl of solubilized PDL cells and then incubating at 37^0C for 30 minutes or until a color change was noted. The reaction was stopped in all samples by the addition of 150 µl of 1 N NaOH. Absorbance was measured at 414 nm using a microtiterplate spectrophotometer (Bio-Rad model 2550 EIA plate reader) and the amount of alkaline phosphatase activity was calculated from a standard curve of para-nitrophenol. The lower limit of detection of the assay was 1.87 nmol/50 µl of para-nitrophenol..

An adaptation of the Pierce microtiter plate BCA protein assay was used to measure cellular protein. Ten µl of 1% triton-X-100 solution was placed well for well in a 96 well microtiter plate. This was followed by the sequential addition of 90 µl of deionized water and 100 µl of BCA reagent. Incubation was accomplished at 60^0C for 60 minutes and the absorbance was read at 570 nm in a microtiterplate spectrophotometer. The amount of protein was calculated from a standard curve constructed from varying concentrations of BSA. The lower limit of detection for the assay was 15 µg/10 µl of protein.

2. <u>Alkaline Phosphatase Histochemical Assay</u>. Cells were plated at a density of 25,000 cells/cm^2 in six wells of a 24 well culture plate (Corning) and grown to confluence. Culture medium was removed and the cultures rinsed one time with HBSS. One ml of test medium was added to three wells. The remaining three wells were stimulated with 2.35×10^{-8} M VitD. All wells were incubated for 48 hours. Medium was removed, cells rinsed once with PBS, and fixed using 2 volumes of 2% Citrate Working Solution to 3 volumes of 60% citrate buffered acetone. Alkaline phosphatase activity was visualized using the Sigma alkaline phosphatase kit #85. Naphthol AS-MX liberated at sites of alkaline phosphatase activity was coupled with either fast violet B or fast Blue RR diazonium salts forming an insoluble, visible pigment. Visual evaluation

of the staining response compared basal activity with activity stimulated by VitD and was scored as increased, decreased or no change.

3. Osteocalcin Assay. Osteocalcin is a protein released during the mineralization of osteoblast differentiation that is used to characterize the osteoblast-like phenotype. Cells were plated at a density of 25,000 cells/cm^2 in six wells of a 6 well tissue culture plate (Corning) and grown to confluence. Culture medium was removed and the cultures rinsed one time with HBSS. Cells were stimulated in triplicate wells with 2 ml of test medium containing 0 nM or 2.35 x 10^{-8} M VitD in DMEM, 0.1% BSA and 1% PSF and then incubated for 24 hours at 37^0C. This medium was removed to waste, replaced with 1.5 ml of medium with or without VitD, and the incubation continued for another 48 hours. The medium was removed and stored in 2 ml microcentrifuge tubes at -20^0C until assayed for osteocalcin using a commercially available human osteocalcin radioimmunoassay kit (BTI, Stoughton, MA).

4. Cyclic AMP Assay. The response to human parathyroid hormone (PTH) was examined by measuring intracellular c-AMP levels. Cells were plated in six wells of a 24 well cell culture plate at a density of 25,000 cells/cm^2 and grown to confluence. The medium was removed and rinsed one time with HBSS. Cells were stimulated in triplicate wells with 0.5 ml of test medium containing 0.1% BSA, 1 mM isobutylmethylxanthine (Sigma), 1% PSF and 0 or 100 ng/ml of PTH, and incubated for 30 minutes at 37^0C. The incubation medium was then discarded and the cell layer rinsed three times with ice cold PBS. C-AMP was then extracted from the cells using an acid/ethanol extraction procedure. A 0.5 ml volume of ice cold 20 mM HCL/100% ethanol solution was added to each well and the plates placed at -20^0C for 18 hours. At the end of this time the acid/ethanol solution was placed in 2 ml microcentrifuge tubes, evaporated to

dryness and placed at -20^{0}C until ready for assay. Immediately prior to assay, the residue in the microcentrifuge tubes was resuspended in assay buffer supplied in a commercially available radioimmunoassay kit (BTI, Stoughton, MA) used for c-AMP quantitation.

5. Mineralization. The mineralization assay was similar to that reported by Boskey *et al.*, (1992). Cells were plated at an initial density of 25,000 cells/cm^2 in 24 well culture plates and grown to confluence. At the beginning of the assay 1 ml of growth medium consisting of DMEM/F12 supplemented with 10% FBS was added to each well. Basal experimental medium included growth medium with additions of calcium chloride to bring the final calcium concentration to 1.1 mM, dibasic potassium phosphate to bring the phosphate concentration to 4 mM and 25 µg/ml of ascorbic acid. Basal experimental medium containing either 100 nM VitD or 100 nM dexamethasone (Sigma), or in which the 4 mM phosphate was replaced with 2.5 mM ß-glycerolphosphate (Sigma) was also tested (Carnes *et al*, 1997). Mineralization of PDL cells grown in basal experimental medium and each modification was evaluated using 6 replicates. Cells were incubated for 21-39 days at 37^{0}C in an atmosphere of 95% air and 5% CO_2 with medium changes every 3-4 days. Mineralization was determined by von Kossa staining (Putt, 1972). Staining over greater than 10% of the surface area of the monolayer was scored as a positive response.

D. Culture of PDL Cells in Semisolid Medium.

As an alternative procedure for selection of osteoblast-like cells, PDL cells were cultured in semisolid medium and evaluated for anchorage-independent growth in the presence of growth factors and hormones. Culture of PDL cells in semisolid medium followed the methods described by Guenther *et al.* (1989) using a three layer agarose system. Initially, 28,000 cells were

suspended in 1 ml of 0.25% liquefied low gel temperature agarose (Difco) prepared in DMEM supplemented with 10% FBS, 1% PSF, 2 ng/ml TGF-ß1 (R&D, Minneapolis, MN) and 5 ng/ml of epidermal growth factor (EGF). The liquid agarose (0.2 ml) was poured into all wells of chilled (4^0C) 24 well plates previously coated with 0.2 ml of 0.6% standard melting agarose (Difco) also prepared in DMEM supplemented with 10% FBS and 1% PSF. These two layered agarose plates were again chilled. After cooling, 0.5 ml DMEM containing 10% FBS, 1% PSF and the growth factors (described above) was layered over the now solidified agarose. Every 2-3 weeks, medium with 10% FBS and 1% PSF was added on top of the agarose system. Colonies which exhibited anchorage independent growth and were larger than 50 μm were transferred individually to 96 well plates. The colonies were then allowed to attach and grow to confluence in DMEM supplemented with 10% FBS and 1% PSF. Cultures were then expanded and characterized using assays previously described. Ideal colony size has been reported to be 90-100 μm (Guenther *et al.*, 1989).

E. <u>Immortalization</u>.

The immortalization protocol was used to establish immortal cell lines from osteoblast-like cells selected and characterized as described above. Cell lines were immortalized to preserve cell populations for future study.

1. <u>Transfection of Cell Populations</u>. Cell populations exhibiting high biochemical alkaline phosphatase activity after first passage were infected for 24-48 hours with the supernatant collected from a 90-100% confluent culture of a defective retrovirus packaging psiCRIP cell line (Danos and Mulligan, 1980) grown in DMEM with 10% FBS and 1% PSF. This cell line produces a retroviral vector carrying a temperature sensitive mutant of the SV-40 large T antigen

inserted in the pZIPtsa58-Neo-SV(X)1 construct (Jat and Sharp, 1989). The viral supernatant was filtered prior to use through a 0.45 mm pore size filter (Millipore). Infections were performed at the permissive temperature of 33^{0}C in 6-well culture plates with 1 ml of supernatant and 8 μg/ml of polybrene (Sigma) to permeablize the target cells. Following infection for 24-48 hours, cultures were changed to selection medium containing 300 μg/ml of neomycin analogue G418 (Sigma) and incubation continued for one month 33^{0}C to select for infected cells. The selection medium was changed every 3-5 days until distinct cell colonies were visible. Cells were trypsinized at confluence and replated for expansion of the infected cell population with media changes every 3-4 days. Each medium change included 300 μg/ml of G418. Following expansion, the cell lines were characterized as previously described at both the permissive temperature of 33^{0}C and at 37^{0}C when the transforming gene is turned off. A human periodontal ligament cell line (HPDL) previously transfected using this same protocol and generously supplied by Dr. Mary MacDougall (University of Texas Health Science Center, San Antonio, TX) was similarly characterized. In addition, this cell line was used to evaluate proliferation of a transformed cell line at both the permissive and non-permissive temperatures.

2. Proliferation Assay of Transformed Cells. Cell proliferation of the HPDL transformed cell line was evaluated at the permissive temperature of 33^{0}C and the non-permissive temperature of 37^{0}C. Cell proliferation was assessed using the reagent WST-1 (Boehringer Mannheim Gmbh, GE) and was based on the conversion of the soluble tetrazolium salt by mitochondrial dehydrogenases to a soluble formazan which absorbs at 450 nm. Cells were plated at either 33^{0}C at a density of 5,000 cells/cm^2 or 37^{0}C (previously grown at 33^{0}C) at a density of 7,000 cells/cm^2 in 96 well cell culture plates in each of three test medium, DMEM, Ca^{++} free

DMEM/F12 and PPP. For incubation at 33^0C, the neomycin analogue G418 was also included in the media. HPDL cells grown in DMEM were used at passage 11 while HPDL cells grown in low-calcium and PPP were used at passage 10. Media changes occurred every 3-4 days. After washing two times with 200µl of test medium, the assay was initiated by adding 100 µl/well of WST-1 and 1 ml of fresh test medium to the plated cells. Following addition of the WST-1 proliferation reagent, cells were incubated for 30 minutes at 33^0C or 37^0C as required. Following incubation, 200 µl of supernatant from each well was plated in duplicate in a separate 96-well culture plate and absorbance was evaluated by a spectrophotometer (Bio-Rad model 2550 EIA plate reader) at a wavelength of 450 nm.. Triplicate wells of each medium were evaluated at nine different time points over a 500 hour time span. Test medium plus WST-1 at the varying volumes served as the blank.

3. Immunohistochemical Antibody Staining of SV-40 T antigen. In order to verify that the transformation construct was incorporated into the genome of the PDL cells, expression of the SV-40 T antigen was evaluated by immunohistochemical staining. Transfected PDL cell populations labeled 13-1 and 16-3 were plated at a density of 7,000 cells/cm^2 in 6 well cell culture plates and incubated at either 33^0C or 37^0C. Subconfluent cells were incubated with a monoclonal antibody to the SV-40 T antigen (AB-2), (Oncogene Research Products, Calbiochem). Specific antibody binding was visualized using a Histostain™ SP Kit (Zymed Laboratories Inc). All procedures were accomplished at room temperature unless otherwise stated. Cells were fixed using 50% methanol for 5 minutes, treated with peroxidase quenching solution (Peroxo-Block, Zymed) for 45 seconds, and rinsed. Cells were then incubated for 10 minutes with 100 µl of serum blocking solution and the excess was removed. Next, 100 µl of the

primary monoclonal antibody, SV40 T-Ag (Ab-2) was applied 3 times, and the cells were incubated at 4^0C overnight. After rinsing with PBS, 100 µl of biotinylated second antibody was applied for 20-30 minutes, followed by rinsing with PBS, and incubated with enzyme conjugate (100 µl) for 10 minutes, rinsed with PBS, and incubated with 100 µl of substrate-chromogen mixture for 8 minutes. Following a distilled water rinse, cells were counterstained using 100 µl of hematoxylin for 30 seconds, rinsed with distilled water, and a coverslip applied using 100 µl of GVA water soluble mounting medium (Biomeda, Foster City, CA).

F. Analysis of Data

All statistical evaluations were carried out using Prism 2 (Graph Pad Software, Inc., San Diego, CA). The alkaline phosphatase response to VitD and PTH was evaluated using ANOVA with Dunnett's procedure for comparing experimental groups to a common control group as the post hoc test. All other analysis involving comparisons between two unpaired groups were accomplished using the t-test. Differences between the median values of VitD stimulated alkaline phosphatase activity or osteocalcin production between DMEM and low-calcium media were determined using the Mann Whitney procedure. Finally, correlation analysis was accomplished using Pearson's (parametric) or Spearman's (non-parametric) procedures.

III. RESULTS

A. Cell Population Characterization

1. Cell Populations. A total of 29 cell populations explanted from PDL (table 1-3) were characterized for the osteoblast-like phenotype. These cell populations were derived from PDL tissue adherent to 49 teeth extracted from 16 different individuals. The specimens were obtained from individuals ranging in age from 15 to 50 years, and included 9 females and 7 males. Six of these individuals contributed erupted teeth that were carious and 2 patients had evidence of periodontal attachment loss. All other patients contributed healthy, erupted teeth. Out of 110 explant cultures initiated, 29 produced vigorous cell outgrowth and were expanded and characterized (26.4%). Cell populations explanted, maintained, and characterized in DMEM medium included 14 of 40 explanted cell populations (35.0%). Cell populations explanted, maintained, and characterized in low-calcium medium included 10 of 30 explanted cell populations (33.3%). Cell populations explanted, maintained, and characterized in PPP were all from the same individual and included 5 of 40 explanted cell populations (12.5%). Proliferation of cell populations following explantation was variable (table 1-3). Average time to reach first passage in a six well cell culture plate was significantly longer for cells grown in PPP compared to cells grown in either DMEM ($p < 0.01$) or low-calcium medium ($p < 0.05$). Cells grown in DMEM and low-calcium medium had similar proliferation times to reach first passage. All three medium had similar expansion times from first passage to fourth passage (table 1-3).

2. Biochemical Alkaline Phosphatase Activity. Alkaline phosphatase is a prominent enzyme expressed early in osteoblast differentiation prior to the initiation of mineralization (Lian

Table 1

PATIENT AND PERIODONTAL LIGAMENT TISSUE CHARACTERISTICS OF CELL POPULATIONS ESTABLISHED IN DULBECCO'S MODIFIED EAGLE'S MEDIUM

Patient	Sex	Age	Teeth	Condition of Teeth	Time to Reach 1st Passage (Days)	Time Between 1st and 4th Passage (Days)
Y-4P4	F	45	#17, #32	Erupted, Healthy	31	38
Y-41P4	F	45	#17, #32	Erupted, Healthy	42	35
1-1P4	F	23	#15, #12	Erupted, Carious	41	28
3-1P4	M	25	#14, #15, #16	Erupted, Carious	28	33
3-2P4	M	25	#14, #15, #16	Erupted, Carious	28	32
5-1P4	M	47	#2, #31	Erupted, Carious	21	27
5-2P4	M	47	#2, #31	Erupted, Carious	21	33
K-1P6	F	50	#1, #3, #4	Erupted, Healthy	25	26
F-5P5	F	30	Full Mouth Extraction (10 teeth)	Erupted, Carious, Periodontitis	21	38
H-5P5	F	42	#32	Erupted, Healthy	16	34
G-3P4	M	37	#1, #16	Erupted, Healthy	16	38
16-1P4	M	15	#5, #12, #21, #28	Erupted, Healthy	31	11
17-2P4	F	20	#5, #12, #21, #28	Erupted, Healthy	24	14
17-5P4	F	20	#5, #12, #21, #28	Erupted, Healthy	20	21
					average = 26.1	average = 29.1

Table 1 lists patient and PDL explant characteristics for 14 cell populations explanted, maintained and characterized in DMEM with 10% FBS. Sex, age, teeth contributing PDL tissue and condition of these teeth at time of explant are described. Population expansion time is reported for the number of days following explant to passage 1 and the number of days from passage 1 to 4. Shaded rows represent cell populations that mineralized in culture. Mineralizing cell populations had significantly longer expansion times ($p < 0.018$) to reach first passage (34.0 days) compared with non-mineralizing cultures maintained in DMEM and low-calcium medium (24.1 days).

Table 2

PATIENT AND PERIODONTAL LIGAMENT TISSUE CHARACTERISTICS OF CELL POPULATIONS ESTABLISHED IN LOW CALCIUM MEDIUM

Patient	Sex	Age	Teeth	Condition of Teeth	Time to Reach 1st Passage (Days)	Time Between 1st and 4th Passage (Days)
B-2P4	F	24	#16, #17, #32	Erupted, Healthy	25	35
D-2P4	M	43	#19	Erupted, Carious, Hypercementosis	24	36
D-5P4	M	43	#19	Erupted, Carious, Hypercementosis	32	33
9-1P6	F	41	#2, #5, #7, #8, #10	#2 Periodontitis, #7, #8, #10 Carious	17	25
9-2P4	F	41	#2, #5, #7, #8, #10	#2 Periodontitis, #7, #8, #10 Carious	21	29
14-4P4	M	35	#16	Erupted, Healthy	44	19
17-1P4	F	20	#5, #12, #21, #28	Erupted, Healthy	24	24
17-4P4	F	20	#5, #12, #21, #28	Erupted, Healthy	20	28
16-3P4	M	15	#5, #12, #21, #28	Erupted, Healthy	28	25
13-1P4	F	23	#1, #16, #17, #32	Erupted, Healthy	18	18
					average = 25.3	average = 27.2

Table 2 lists patient and PDL explant characteristics for 10 cell populations explanted, maintained and characterized in low-calcium medium with 10% FBS. Sex, age, teeth contributing PDL tissue and condition of these teeth at time of explant are described. Population expansion time is reported for the number of days following explant to passage 1 and the number of days from passage 1 to 4. Cell population D-5P4 in the shaded row mineralized in culture.

Table 3

PATIENT AND PERIODONTAL LIGAMENT TISSUE CHARACTERISTICS OF CELL POPULATIONS ESTABLISHED IN PLATELET POOR PLASMA MEDIUM

Patient	Sex	Age	Teeth	Condition of Teeth	Time to Reach 1st Passage (Days)	Time Between 1st and 4th Passage (Days)
M-1P4	M	31	#16	Erupted, Healthy	33	26
M-3P5	M	31	#16	Erupted, Healthy	41	21
M-5P4	M	31	#16	Erupted, Healthy	33	31
M-6P5	M	31	#16	Erupted, Healthy	33	33
M-11P4	M	31	#16	Erupted, Healthy	36	33
					average = 35.2	average = 28.8

Table 3 lists patient and PDL explant characteristics for 5 cell populations explanted, maintained and characterized in DMEM with 5% PPP. Sex, age, teeth contributing PDL tissue and condition of these teeth at time of explant are described. Population expansion time is reported for the number of days following explant to passage 1 and the number of days from passage 1 to 4. Cell populations maintained in PPP had significantly longer expansion times from explant to passage 1 when compared to DMEM ($p < 0.01$) and low-calcium medium ($p < 0.05$).

and Stein, 1992). This enzyme has also be reported in PDL cell cultures (Kawase *et al.*, 1988; Somerman *et al.*, 1988; Piche *et al.*, 1989a; Nojima *et al.*, 1990; Ogata *et al.*, 1995; Goseki *et al.*, 1995; Giannopoulou and Cimasoni, 1996; Carnes *et al.*, 1997; Liu *et al.*, 1997). In the osteoblast, alkaline phosphatase enzyme activity is increased upon stimulation with VitD and decreased upon stimulation with PTH. Alkaline phosphatase activity was present in 28 of 29 PDL cell populations characterized in this study (figure 1). Cell population G-3P4 had high basal levels exceeding the detection limit of the assay and therefore was not used in analysis. Basal alkaline phosphatase activity for cell populations grown in DMEM ranged from 13-1471 pmol/min with a median of 407 pmol/min. Basal levels for cell populations grown in low-calcium medium ranged from 0-692 pmol/min with a median of 216.5 pmol/min. Basal alkaline phosphatase levels for cells grown in PPP medium ranged from 21-89 pmol/min with a median of 48 pmol/min. Comparisons of basal alkaline phosphatase activity between cultures established in the three test medium could not be made due to the variable number of cells in each cell population. Specific activity of alkaline phosphatase was not determined since the protein content in the individual wells of the 96 well plate was below the lower limit of detection for the protein assay. It was, therefore, decided to compare alkaline phosphatase activity in the different test medium using the ratio of hormonal stimulated activity to basal activity (fold stimulation). Twenty-five of 28 PDL cell populations responded with a significant increase in alkaline phosphatase activity when modulated by 2.35×10^{-8}M VitD (figure 1). Only cell populations B-2P4, D-2P4 and 9-2P4 explanted and maintained in low-calcium media did not respond to VitD stimulation. Figure 2 illustrates the increase in alkaline phosphatase activity in response to VitD expressed as stimulated over basal. Data is expressed this way since the magnitude of the values for alkaline phosphatase

Figure 1. Basal and $1,25(OH)_2D_3$ Stimulated Alkaline Phosphatase Activity

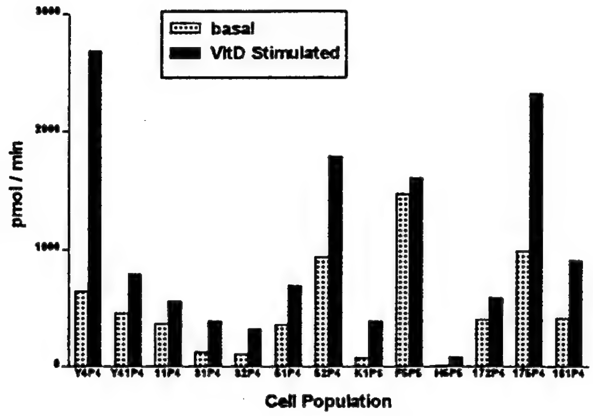
Top. All 13 cell populations established and maintained in DMEM expressed basal levels of alkaline phosphatase activity ranging from 13-1471 pmol/min. All cell populations responded with significant increases ($p < 0.05$) in $1,25(OH)_2D_3$-stimulated alkaline phosphatase activity.

Middle. Nine of 10 cell populations established in low calcium medium expressed basal levels of alkaline phosphatase activity ranging from 33-692 pmol/min. All cell populations except B-2P4, D-2P4 and 9-2P4 responded with significant increases ($p < 0.05$) in $1,25(OH)_2D_3$-stimulated alkaline phosphatase activity.

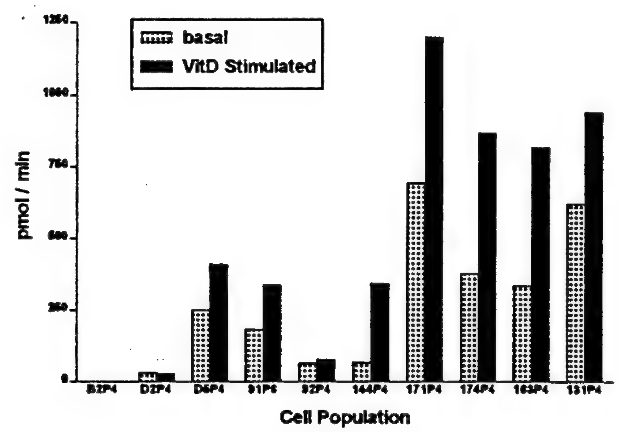
Bottom. All six cell populations established in platelet poor plasma medium expressed basal levels of alkaline phosphatase activity ranging from 21-89 pmol/min. All cell populations had significant increases ($p < 0.05$) in $1,25(OH)_2D_3$-stimulated alkaline phosphatase activity.

Alkaline Phosphatase

PDL Cells Explanted in DMEM



PDL Cells Explanted in Low Calcium Medium



PDL Cells Explanted in Platelet Poor Plasma

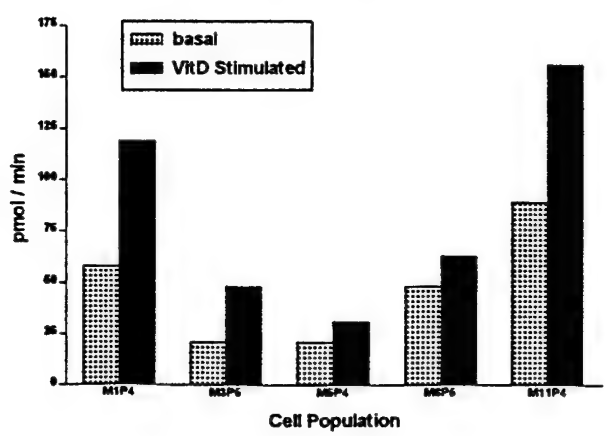


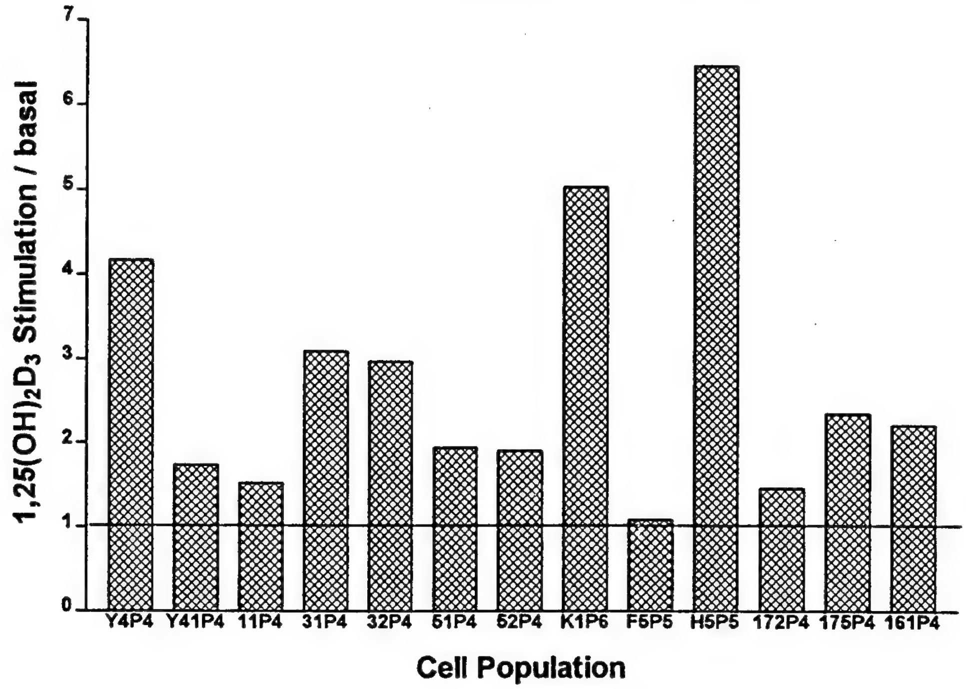
Figure 2. Ratio of $1,25(OH)_2D_3$ Fold Stimulated Alkaline Phosphatase Activity to Basal Activity

Top. All 13 cell populations established and maintained in DMEM responded to $1,25(OH)_2D_3$ stimulation with significant increase in alkaline phosphatase activity over basal levels. Seven of 13 cell populations had a greater than 2-fold stimulation. Those cell populations above the horizontal line had positive fold stimulations greater than one.

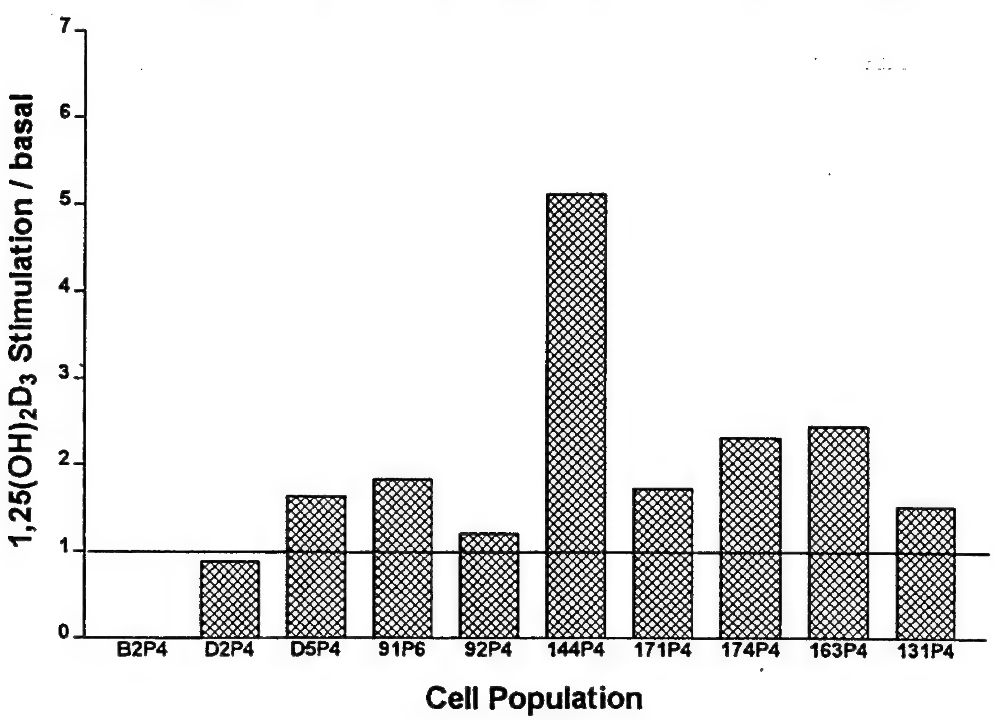
Bottom. Seven of 10 cell populations established in low calcium medium responded to $1,25(OH)_2D_3$ stimulation with significant increases in alkaline phosphatase activity over basal levels. Cell populations D-2P4 and 9-2P4 did not respond to $1,25(OH)_2D_3$ stimulation, while cell population B-2P4 displayed neither baseline or $1,25(OH)_2D_3$–stimulated activity. Three of the 7 responding cell populations displayed greater than 2-fold stimulation. Those cell populations above the horizontal line had positive fold stimulations greater than one.

Alkaline Phosphatase

PDL Cells Explanted in DMEM



PDL Cells Explanted in Low Calcium Medium



activity were different for the two media and protein values were unavailable. When expressed this way, the data shows that cells explanted and maintained in the two media were stimulated to the same degree. Stimulation with 100 ng/ml of PTH resulted in a significant decrease in alkaline phosphatase activity compared to basal levels ($p < 0.05$) for 5/13 cell populations cultured in DMEM and 3/10 cell populations cultured in low-calcium medium (figure 3). PTH had no effect on the alkaline phosphatase activity of cell populations cultured in PPP medium (figure 3). Figure 4 illustrates the decrease in alkaline phosphatase activity in response to PTH expressed as stimulated over basal. Significant hormonal modulation of alkaline phosphatase activity in response to both VitD and PTH was seen in 4/13 cell populations cultured in DMEM, 2/10 cell populations cultured in low-calcium medium and 0/5 cell populations cultured in PPP medium.

3. <u>Histochemical Alkaline Phosphatase Activity</u>. Visual comparison of basal and VitD stimulated alkaline phosphatase activity was accomplished using histochemical methods. Histochemical alkaline phosphatase activity was characterized as increased, decreased or no change upon modulation with VitD. Alkaline phosphatase activity evaluated histochemically was consistent with the results noted biochemically for cells cultured in each of the three test media. While the biochemical assay can quantitate enzyme activity, it cannot evaluate distribution of alkaline phosphatase producing cells. Distribution of responding cells can be evaluated histochemically. It was determined that the distribution of basal and VitD stimulated alkaline phosphatase activity was uniformly distributed over the entire cell population. The increase in VitD stimulated alkaline phosphatase activity compared to basal activity is displayed for a representative cell population in figure 5. An evenly distributed increase in stained cells is apparent following stimulation by VitD.

Figure 3. Basal and PTH Stimulated Alkaline Phosphatase Activity

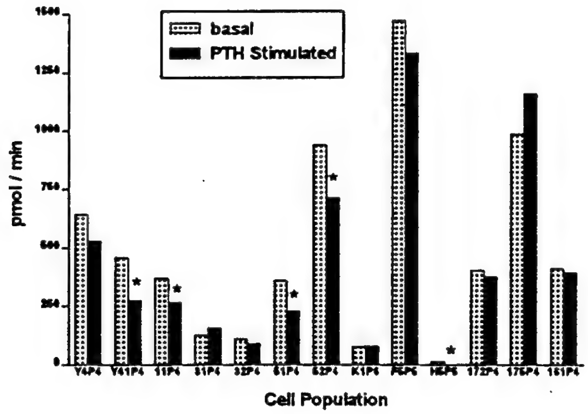
Top. All 13 cell populations established and maintained in DMEM expressed basal levels of alkaline phosphatase activity ranging from 13-1471 pmol/min. Five cell populations (designated with an *) responded with significant decreases ($p < 0.05$) in alkaline phosphatase activity when stimulated with PTH (100 ng/ml).

Middle. Nine of 10 cell populations established in low calcium medium expressed basal levels of alkaline phosphatase activity ranging from 33-692 pmol/min. Three cell populations (designated with an *) responded with significant decreases ($p < 0.05$) in alkaline phosphatase activity when stimulated with PTH (100 ng/ml).

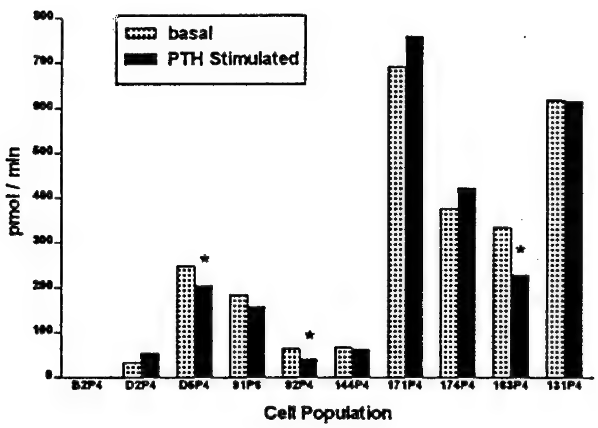
Bottom. All six cell populations established in platelet poor plasma medium expressed basal levels of alkaline phosphatase activity ranging from 21-89 pmol/min. No cell populations responded with significant decreases in alkaline phosphatase activity when stimulated with PTH (100 ng/ml).

Alkaline Phosphatase

PDL Cells Explanted in DMEM



PDL Cells Explanted in Low Calcium Medium



PDL Cells Explanted in Platelet Poor Plasma

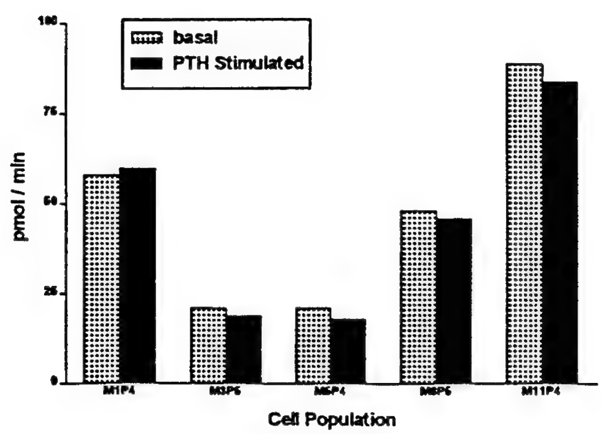
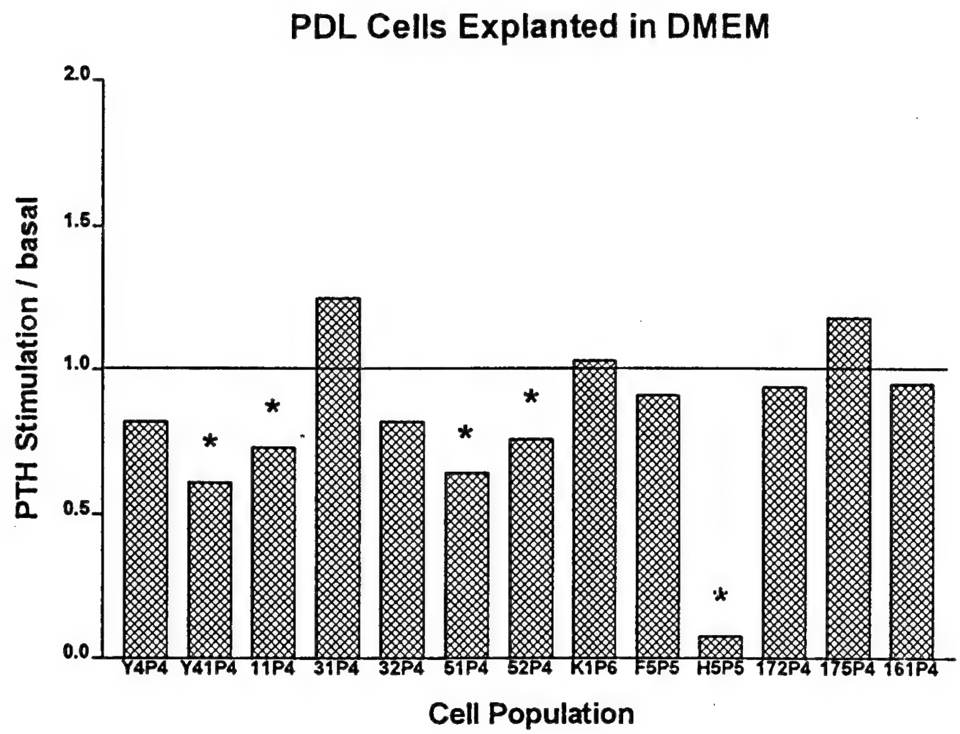


Figure 4. Ratio of PTH Stimulated Alkaline Phosphatase Activity to Basal Activity.

Top. Five of 13 cell populations established and maintained in DMEM responded with significant decreases (* $p < 0.05$) in alkaline phosphatase activity when stimulated with PTH (100 ng/ml). Those cell populations below the horizontal line had percent fold decreases below one.

Bottom. Three of 10 cell populations established and maintained in low calcium medium responded with significant decreases (* $p < 0.05$) in alkaline phosphatase activity when stimulated with PTH (100 ng/ml). Those cell populations below the horizontal line had percent fold decreases below one.

Alkaline Phosphatase



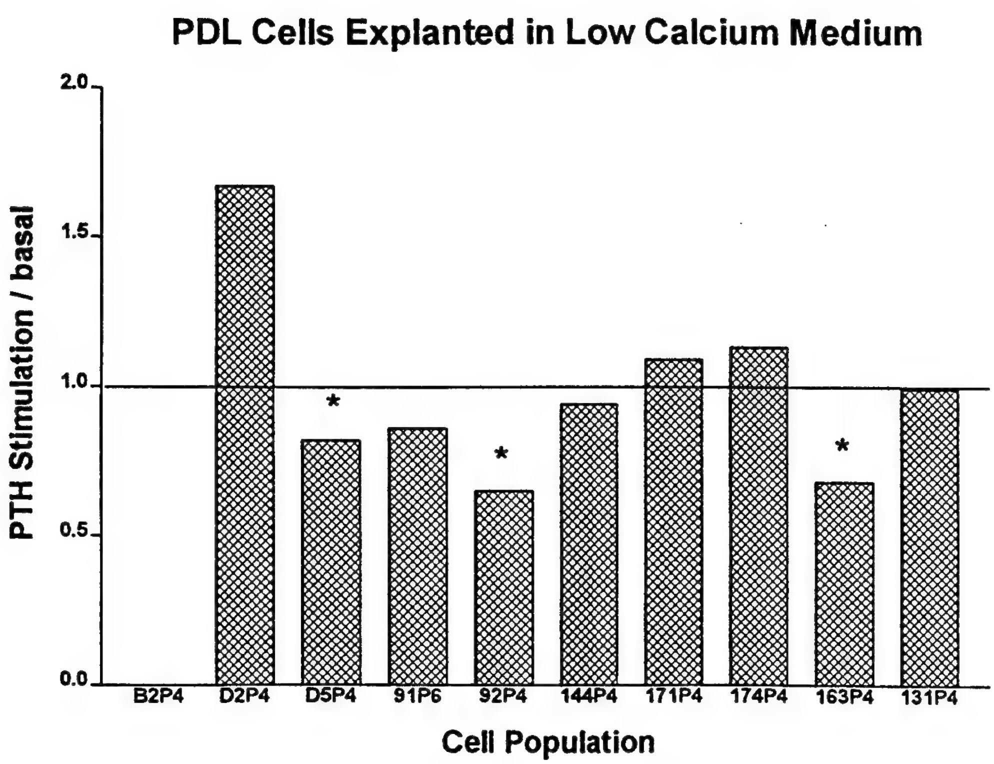


Figure 5. Photomicrographs of Representative Histochemical Evaluation for Alkaline Phosphatase Activity

Figure A. Representative photomicrograph of basal level of alkaline phosphatase activity in cell population HPDL P6. Substrate for the alkaline phosphatase reaction was fast violet B salt. Original magnification = 25X.

Figure B. Representative photomicrograph of $1,25(OH)_2D_3$-stimulated level of alkaline phosphatase activity in cell population HPDL P6. Substrate for the alkaline phosphatase reaction was fast violet B salt. Original magnification = 25X.

4. Osteocalcin Production. Osteocalcin is an extracellular matrix protein expressed in the mature osteoblast (Lian and Stein, 1992). Synthesis and secretion of this protein has been reported for populations of PDL cells as well (Nojima *et al.*, 1990; Carnes *et al.*, 1997). Because of limitations on the number of cells evaluated per well, and the sensitivity of the assay, basal osteocalcin production could not be quantitated for most PDL cell populations. Therefore, only VitD-stimulated osteocalcin production is reported (figure 6). In cell populations cultured in DMEM, stimulated osteocalcin production ranged from non-detectable levels in cell populations H-5P5 and G-3P4 to a maximum of 5.3 ng/ml for cell population 16-1P4 with a median of 1.6 ng/ml. In cells cultured in low-calcium medium, levels ranged from non-detectable to 5.2 ng/ml with a median of 0.5 ng/ml. For cells cultured in PPP medium, the range was between non-detectable to 1.6 ng/ml with a median of 0.3 ng/ml. A response to VitD was seen in 11/13 cell populations cultured in DMEM, 6/10 cell populations cultured in low-calcium medium and 1/5 cell populations cultured in PPP medium. Response was defined as a detectable level of osteocalcin (> 0.35 ng/ml), since basal levels were not detectable.

5. Cyclic AMP Production. Osteoblasts respond to PTH with an increase in intracellular cAMP levels. An increase in cAMP levels following stimulation by PTH has been reported for PDL cell populations (Piche *et al.*, 1989; Nohuctu *et al.*, 1995). In the present study, basal levels of cAMP ranged between 0.1 to 15.2 pmol/ml with a median of 3.1 pmol/ml for cell populations cultured in DMEM. Basal levels ranged from 0.3-9.6 pmol/ml with a median of 1.6 pmol/ml for cell populations cultured in low-calcium medium. Cells cultured in PPP medium had basal levels of cAMP ranging from 0.6-1.8 pmol/ml with a median of 1.0 pmol/ml. A significant increase in the level of cAMP following stimulation by PTH ($p < 0.05$) occurred in 7/13 cell populations

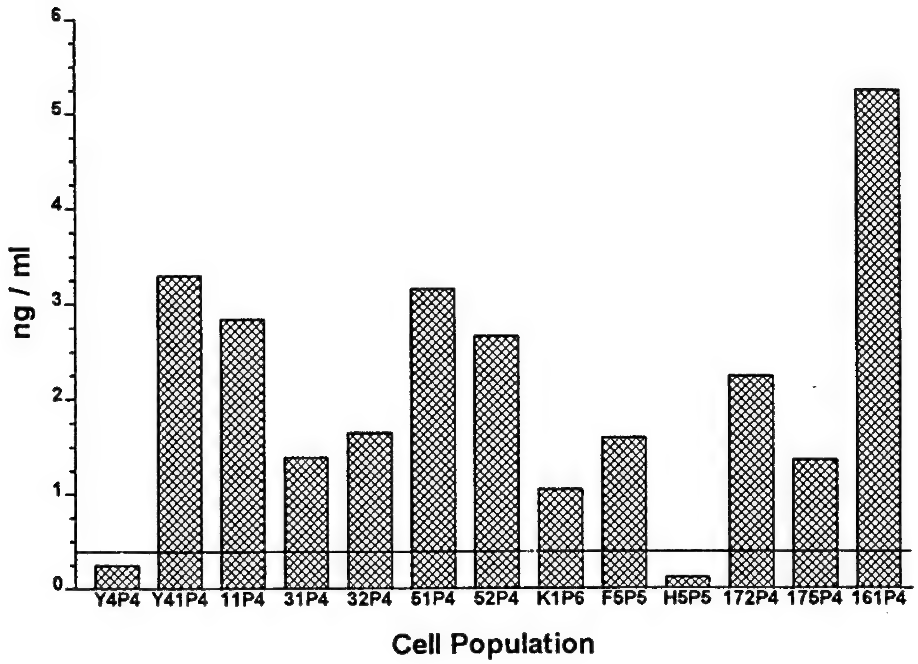
Figure 6. $1,25(OH)_2D_3$ Stimulated Osteocalcin

Top. Eleven of 13 cell populations explanted and maintained in DMEM responded with detectable levels of osteocalcin in response to $1,25(OH)_2D_3$. Osteocalcin levels ranged from 1.1 to 5.3 ng/ml. Limit of detection was 0.35 ng/ml and is indicated on graph by the horizontal line.

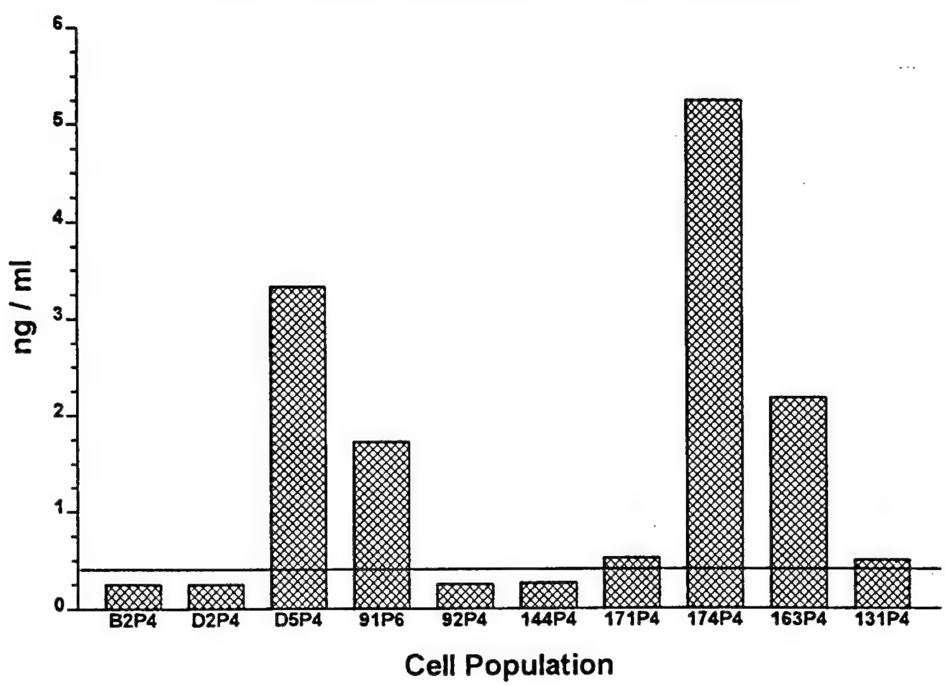
Bottom. Six of 10 cell populations explanted and maintained in low calcium medium responded with detectable levels of osteocalcin in response to $1,25(OH)_2D_3$. Osteocalcin levels ranged from 0.5 to 5.2 ng/ml. Limit of detection was 0.35 ng/ml and is indicated on graph by the horizontal line.

$1,25(OH)_2D_3$-Stimulated Osteocalcin

PDL Cells Explanted in DMEM



PDL Cells Explanted in Low Calcium



cultured in DMEM, 4/10 cell populations cultured in low-calcium medium, and 3/5 cell populations grown in PPP medium (data not shown).

6. Mineralization. The hallmark of a fully differentiated osteoblast is the production of mineralized tissue. Therefore, it was of interest to evaluate whether or not the osteoblast-like cell populations characterized in the present study were capable of mineralization. Mineralization occurred in 3/13 cell populations cultured in DMEM (Y-41P4, 1-1P4 and 5-1P4) and 1/10 cell populations cultured in low-calcium medium (D-5P4). None of the cell populations established in PPP medium mineralized in culture. All cultures were evaluated for mineralization during long term culture ranging from 21-39 days. Of the mineralizing conditions evaluated, mineralization only occurred when 2.5 mM β-glycerophosphate was present in the experimental medium. A photograph representative of the mineralization response as evaluated by Von Kossa staining is displayed in figure 7. Cell populations which mineralized were derived from explants which took significantly longer times ($p < 0.018$) to reach confluence and first passage when compared to their non-mineralizing counterparts explanted and maintained in DMEM or low-calcium medium (table 1 and 2). However, no differences were apparent in the rate of proliferation between mineralizing and non-mineralizing cell populations during subsequent passages.

7. Comparisons of Different Cell Culture Media on Selection of the Osteoblast-Like Phenotype. It was of interest to ascertain whether or not there was any advantage to using DMEM or low-calcium medium for establishing cultures enriched in the osteoblast-like phenotype. Because all cell populations established in PPP medium were from the same individual, and mineralization was not apparent, this medium was not considered for analysis. In order to identify any advantage of DMEM or low-calcium medium, both VitD stimulated alkaline

Figure 7. Representative Mineralization Observed Following Von Kossa Histochemical Stain

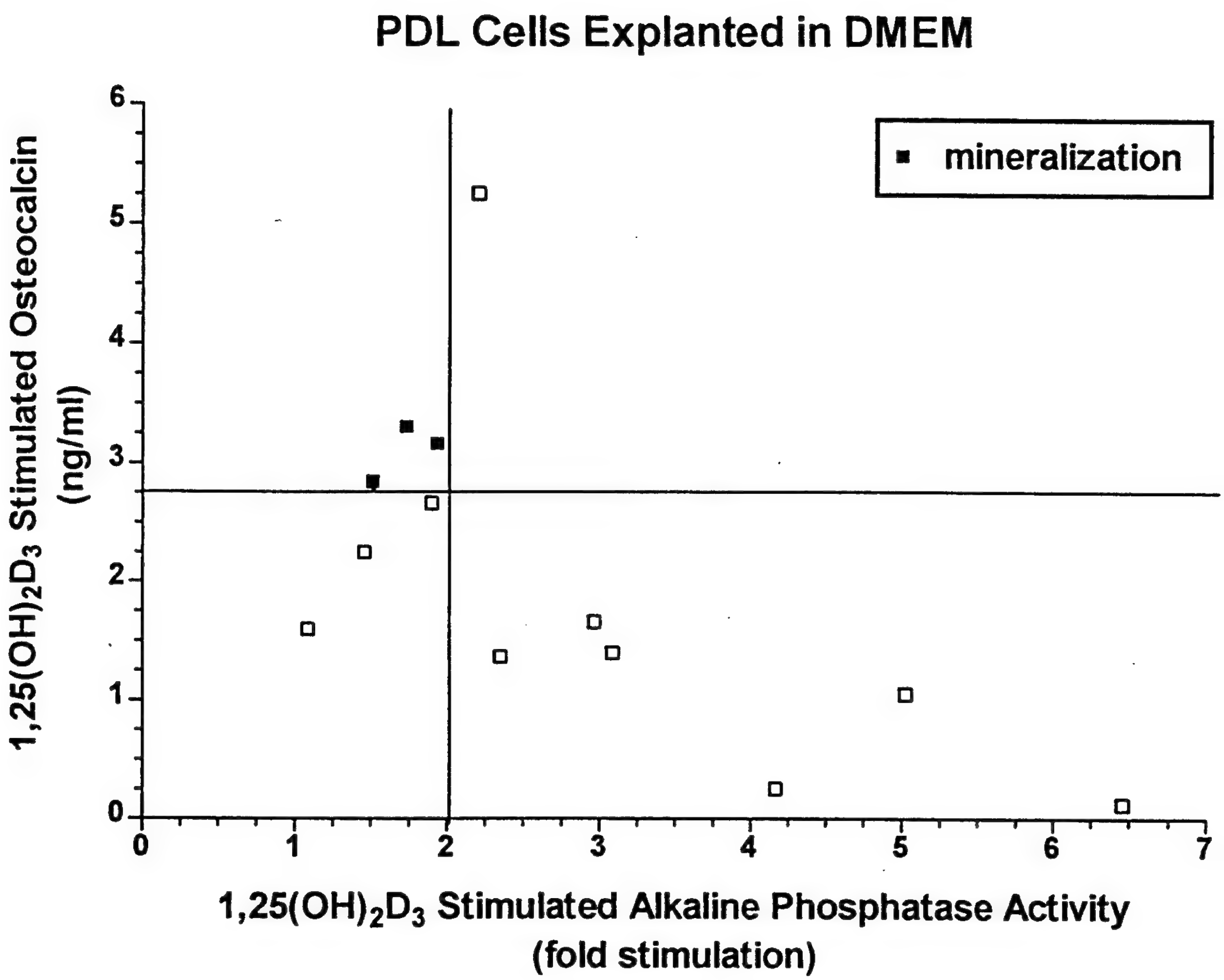
Photograph of cell population Y-41 explanted and maintained in DMEM. Significant mineralization occurred after fourth passage when cells were cultured in the presence of 2.5 mM β-glycerophosphate for 30 days (well on the right). No mineralization was apparent in the absence of β-glycerophosphate (well on the left).

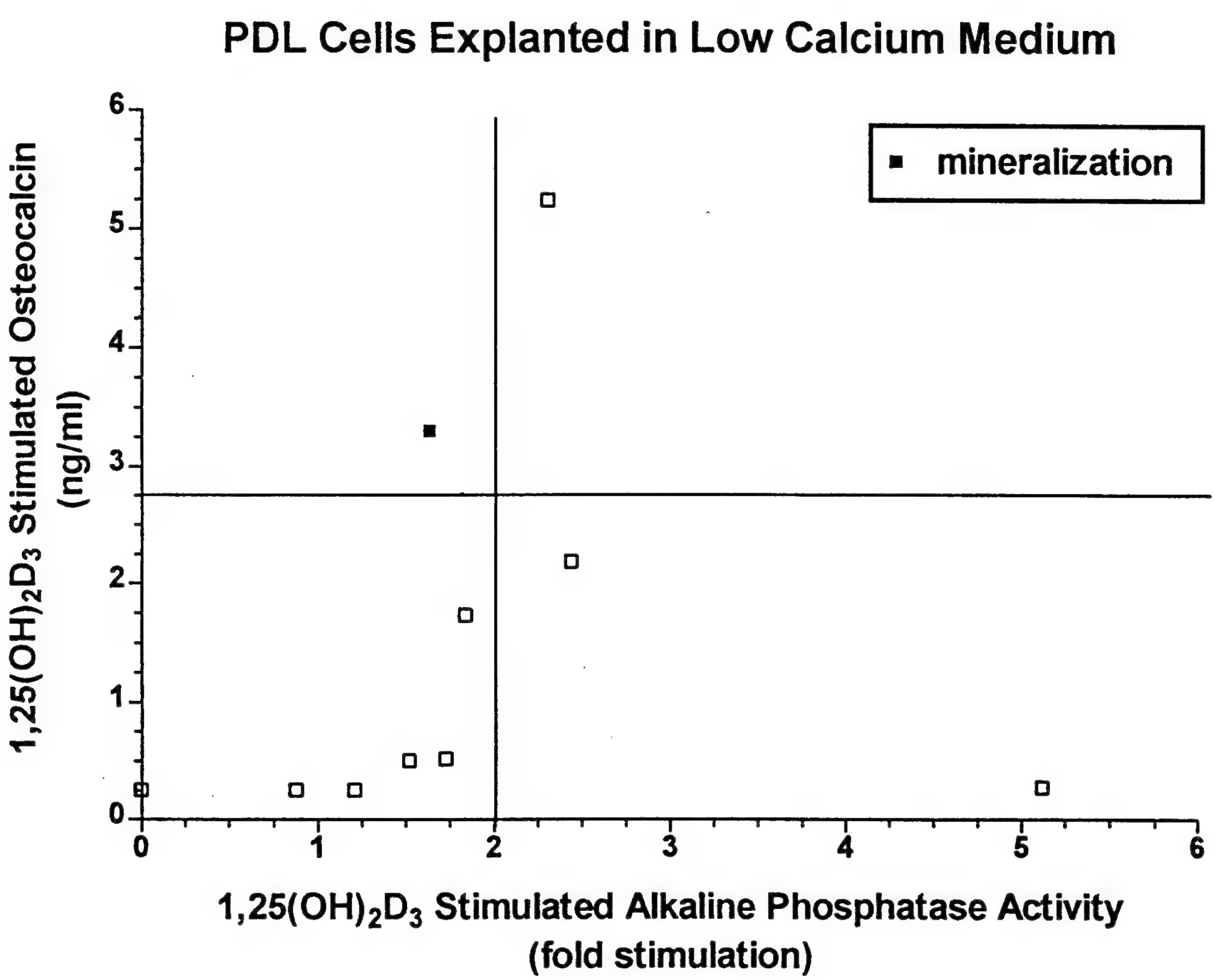
phosphatase activity and VitD stimulated osteocalcin production of cell populations established in these two media were compared. No significant differences were ascertained between either the mean or median values for these parameters between the cell populations established in the two culture media. It was reasoned that if one of these media proved advantageous for the selection of osteoblast-like cells, a significant difference would have been apparent between the media.

8. Markers for an Osteoblast-Like Phenotype. The hallmark of a fully differentiated osteoblast is the production of a mineralized matrix. As noted above, three cell populations explanted and maintained in DMEM mineralized as indicated by the presence of Von Kossa positive material (figure 7). Vitamin D stimulated alkaline phosphatase activity and osteocalcin production are also characteristic of the osteoblast-like phenotype. When VitD stimulated osteocalcin production was plotted on a scatter diagram against VitD stimulated alkaline phosphatase activity (expressed as stimulated/basal), the 3 cell populations that mineralized clustered together. These three cell populations were isolated arbitrarily on the diagram by drawing horizontal and vertical lines parallel to the x and y axes respectively (figure 8). Compared to all other cell populations explanted and maintained in DMEM, only these mineralizing populations responded to VitD with osteocalcin production greater than 2.75 ng/ml and stimulation of alkaline phosphatase activity less than 2-fold. When these same parameters were evaluated using a similar plotting strategy for cell populations explanted and maintained in low-calcium medium, the one cell population which mineralized fell within the same quadrant of the diagram (figure 9) as did the 3 cell populations which mineralized in DMEM. No cell populations explanted and maintained in PPP medium mineralized and none of these populations fell within the quadrant of the graph which contained mineralizing populations from the other

Figure 8. Mineralization Verses $1,25(OH)_2D_3$ Stimulated Osteocalcin and Alkaline Phosphatase Activity in DMEM

Scatter diagram plotting $1,25(OH)_2D_3$-stimulated/basal alkaline phosphatase activity verses $1,25(OH)_2D_3$-stimulated osteocalcin for cell populations explanted and maintained in DMEM. No significant correlation existed between $1,25(OH)_2D_3$-stimulated levels of alkaline phosphatase activity and osteocalcin production ($r = -0.46$). Cell populations which mineralized are identified by the solid squares. These cell populations were isolated from the other cell populations on the plot using arbitrary lines drawn parallel to the x and y axes. These arbitrary lines represent a 2 fold stimulation of alkaline phosphatase activity and 2.75 ng/ml of $1,25(OH)_2D_3$-stimulated osteocalcin production.


PDL Cells Explanted in DMEM
mineralization
1,25(OH)2D3 Stimulated Osteocalcin (ng/ml)
1,25(OH)2D3 Stimulated Alkaline Phosphatase Activity (fold stimulation)
0
1
2
3
4
5
6
7


PDL Cells Explanted in Low Calcium Medium
mineralization
$1,25(OH)_2D_3$ Stimulated Osteocalcin (ng/ml)
$1,25(OH)_2D_3$ Stimulated Alkaline Phosphatase Activity (fold stimulation)
0
1
2
3
4
5
6

media (figure 10). Despite the fact that the mineralizing cell populations were associated with particular levels of VitD stimulated alkaline phosphatase and osteocalcin production, there was no statistical correlation between these two parameters for cell populations explanted in either DMEM or low-calcium (DMEM $r = -0.46$; low-calcium $r = 0.12$).

Using these same parameters to define mature osteoblast-like cell populations, other characteristics of cells capable of producing a mineralized matrix were evaluated. Regardless of media in which they were explanted, all cell populations which mineralized responded to PTH with a significant decrease in alkaline phosphatase activity (figures 11 and 12). Three of 4 populations also responded to PTH stimulation with increases in intracellular cAMP (figures 11 and 12). Response of the non-mineralizing populations to stimulation with PTH was variable, with no discernible correlations.

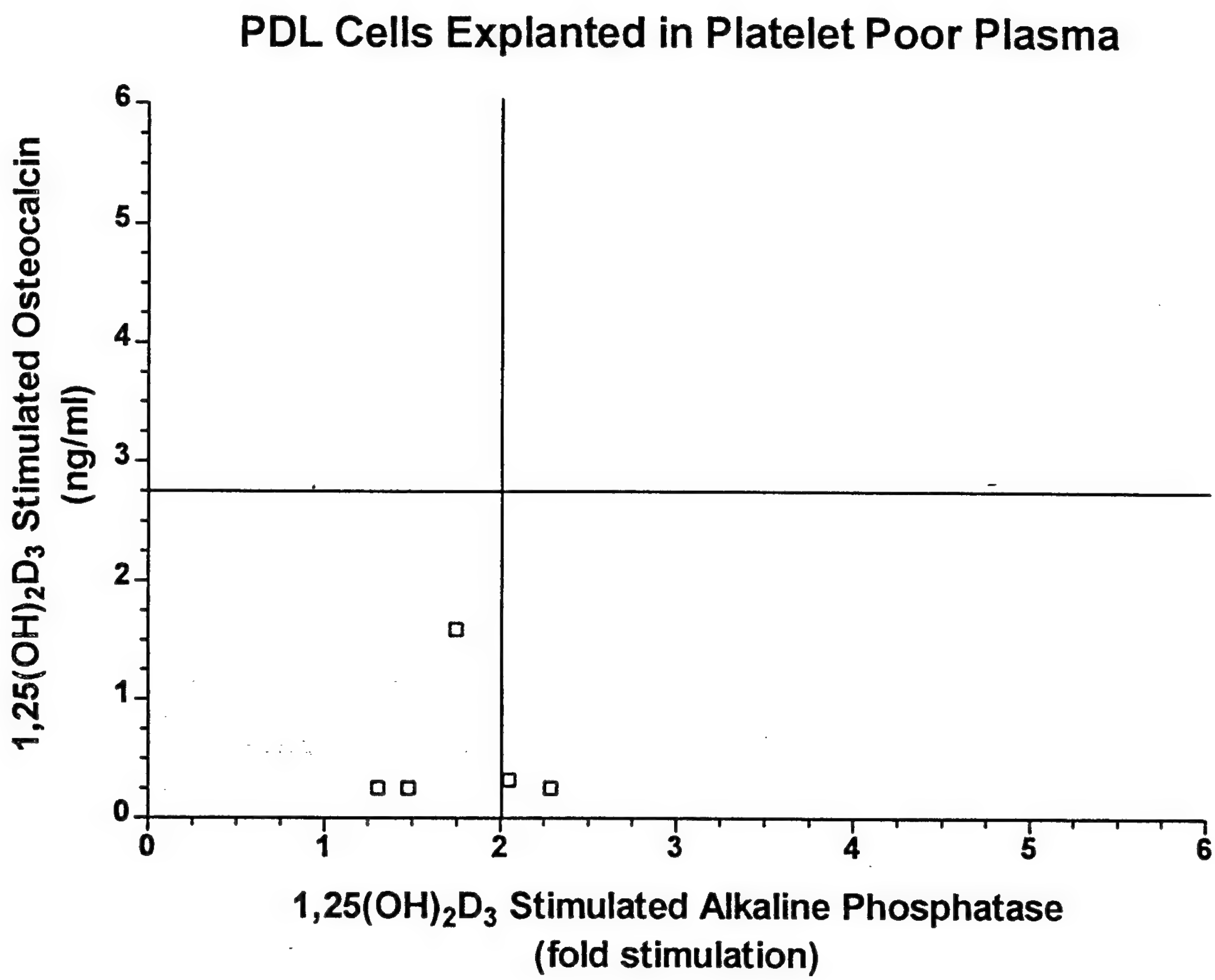
In order to determine whether or not age and sex of the donor of the PDL tissue had any influence on the phenotype expressed by the various cell populations, these parameters were also evaluated on the scatter diagrams above. There were no apparent correlations between the age and sex of the donor contributing PDL tissue and the expression of phenotype in any of the culture media. The distribution of male and female as well as persons over or under 35 years of age was variable (figures 13 and 14). Age 35 was chosen as the break point since it was the median age for cultures established in DMEM.

B. Immortalization of PDL Cell Populations

1. Characterization of the Transformed Cell Population HPDL. It has been reported that when cells transformed with the SV-40 large T antigen - temperature sensitive construct used in this study are moved from the permissive temperature of 33^0C to a higher temperature (39^0C),

Figure 10. Mineralization Verses $1,25(OH)_2D_3$ Stimulated Osteocalcin and Alkaline Phosphatase Activity in Platelet Poor Plasma Medium

Scatter diagram plotting $1,25(OH)_2D_3$-stimulated/basal alkaline phosphatase activity verses $1,25(OH)_2D_3$-stimulated osteocalcin for cell populations explanted and maintained in platelet poor plasma medium. Arbitrary lines determined in figure 8, representing a 2 fold stimulation of alkaline phosphatase activity and 2.75 ng/ml of $1,25(OH)_2D_3$-stimulated osteocalcin production, were drawn on the plot. No cell populations mineralized in platelet poor plasma medium, and no cell populations plotted in the upper left quadrant of the plot.



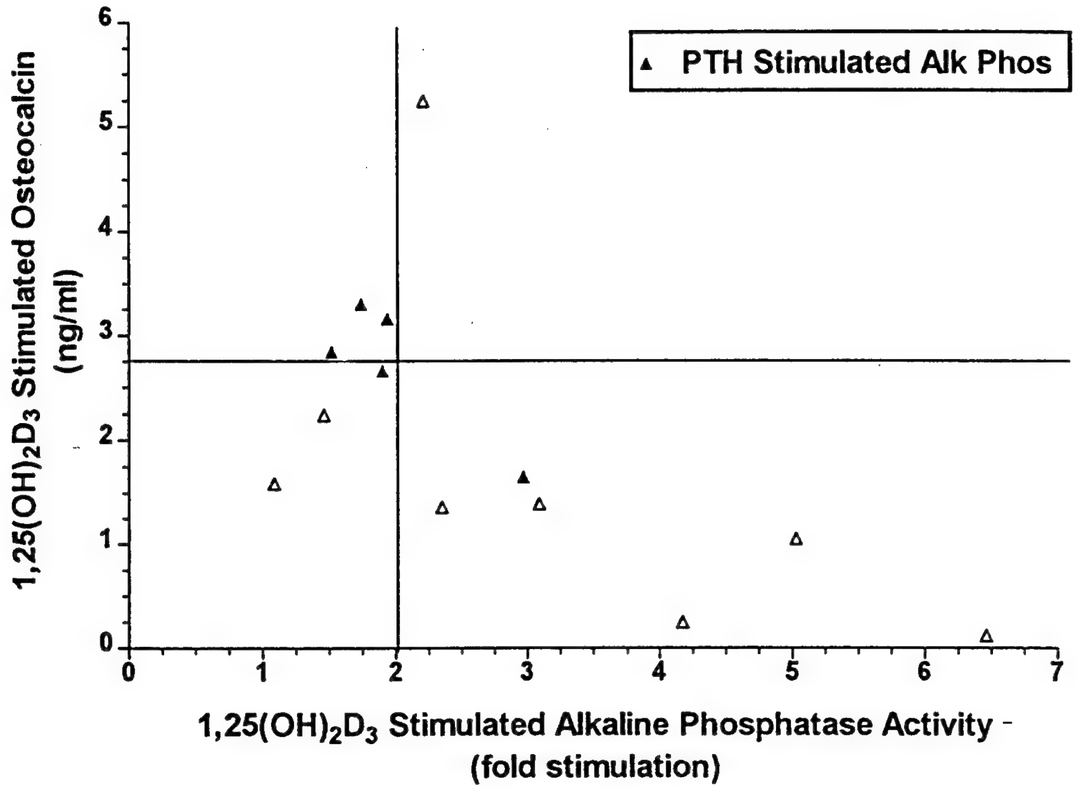
PDL Cells Explanted in Platelet Poor Plasma
1,25(OH)2D3 Stimulated Osteocalcin (ng/ml)
0
1
2
3
4
5
6
1,25(OH)2D3 Stimulated Alkaline Phosphatase (fold stimulation)

Figure 11. Parathyroid Hormone Stimulation Verses $1,25(OH)_2D_3$ Stimulated Osteocalcin and Alkaline Phosphatase Activity in DMEM

Top. Scatter diagram plotting $1,25(OH)_2D_3$-stimulated/basal alkaline phosphatase activity verses $1,25(OH)_2D_3$-stimulated osteocalcin for cell populations explanted and maintained in DMEM. Arbitrary lines representing a 2 fold stimulation of alkaline phosphatase activity and 2.75 ng/ml of $1,25(OH)_2D_3$-stimulated osteocalcin production was drawn on the plot with those cell populations which mineralized plotting in the upper left quadrant of the diagram. Cell populations with significant PTH-stimulated alkaline phosphatase activity are depicted using solid triangles with all 3 cell populations which mineralized presenting with a significant response. The remaining cell populations did not respond to PTH (open triangles).

Bottom. Scatter diagram plotting $1,25(OH)_2D_3$-stimulated/basal alkaline phosphatase activity verses $1,25(OH)_2D_3$-stimulated osteocalcin for cell populations explanted and maintained in DMEM. Arbitrary lines representing a 2 fold stimulation of alkaline phosphatase activity and 2.75 ng/ml of $1,25(OH)_2D_3$-stimulated osteocalcin production was drawn on the plot with those cell populations which mineralized plotting in the upper left quadrant of the diagram. Cell populations with significant PTH-stimulated cAMP production are depicted using solid circles with 2 of 3 cell populations which mineralized presenting with a significant response. The remaining cell populations did not respond to PTH (open circles).

PDL Cells Explanted in DMEM



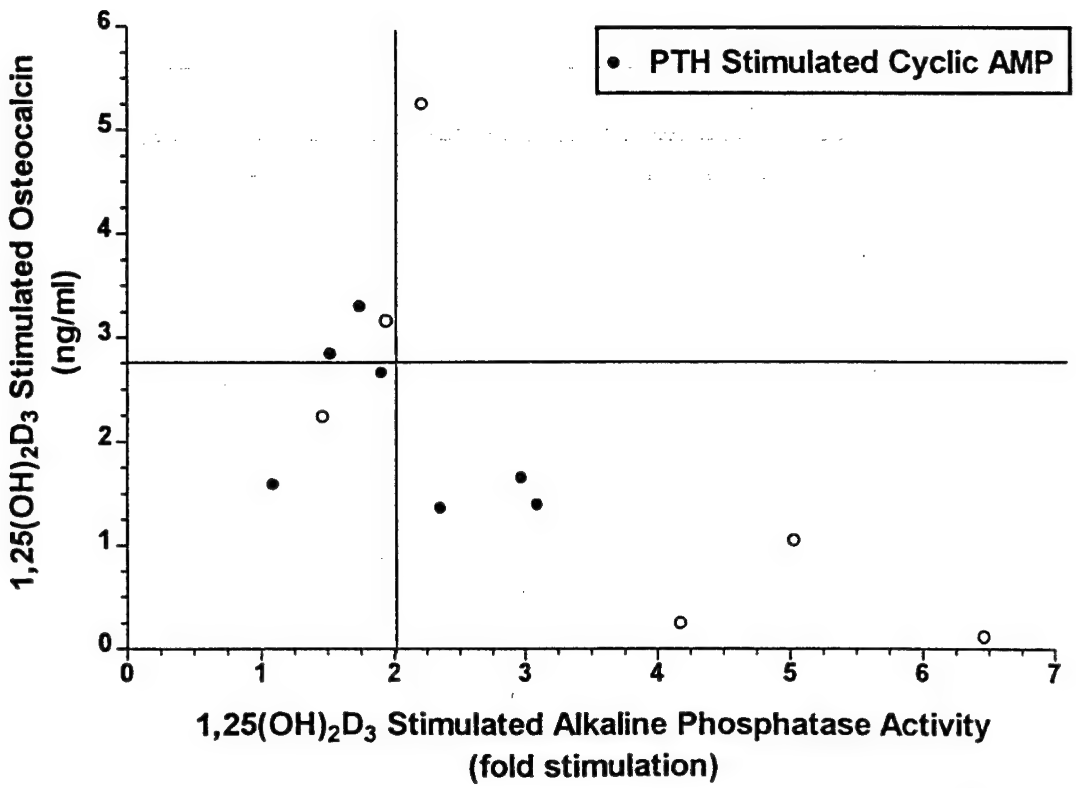
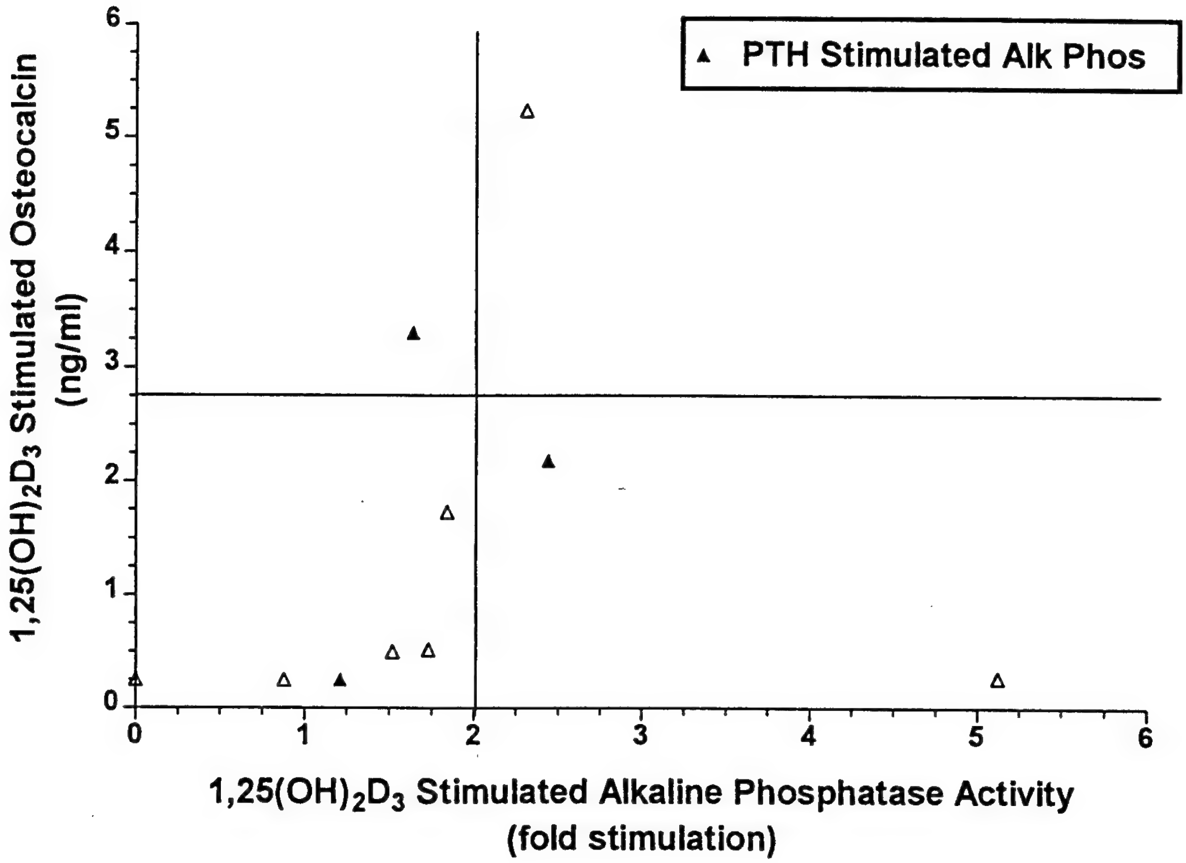


Figure 12. Parathyroid Hormone Stimulation Verses $1,25(OH)_2D_3$ Stimulated Osteocalcin and Alkaline Phosphatase Activity in Low Calcium Medium

Top. Scatter diagram plotting $1,25(OH)_2D_3$-stimulated/basal alkaline phosphatase activity verses $1,25(OH)_2D_3$-stimulated osteocalcin for cell populations explanted and maintained in low calcium medium. Arbitrary lines representing a 2 fold stimulation of alkaline phosphatase activity and 2.75 ng/ml of $1,25(OH)_2D_3$-stimulated osteocalcin production were drawn on the plot with cell population D-5P4 which mineralized plotting in the upper left quadrant of the diagram. Cell populations with significant PTH-stimulated alkaline phosphatase activity are depicted with cell population D-5P4 presenting with a significant response. The other cell populations did not respond to PTH (open triangles).

Bottom. Scatter diagram plotting $1,25(OH)_2D_3$-stimulated/basal alkaline phosphatase activity verses $1,25(OH)_2D_3$-stimulated osteocalcin for cell populations explanted and maintained in low calcium medium. Arbitrary lines representing a 2 fold stimulation of alkaline phosphatase activity and 2.75 ng/ml of $1,25(OH)_2D_3$-stimulated osteocalcin production were drawn on the plot with cell population D-5P4 which mineralized plotting in the upper left quadrant of the diagram. Cell populations with significant PTH-stimulated cAMP production are depicted using solid circles with cell population D-5P4 presenting with a significant response. The other cell populations did not respond to PTH (open circles).

PDL Cells Explanted in Low Calcium Medium



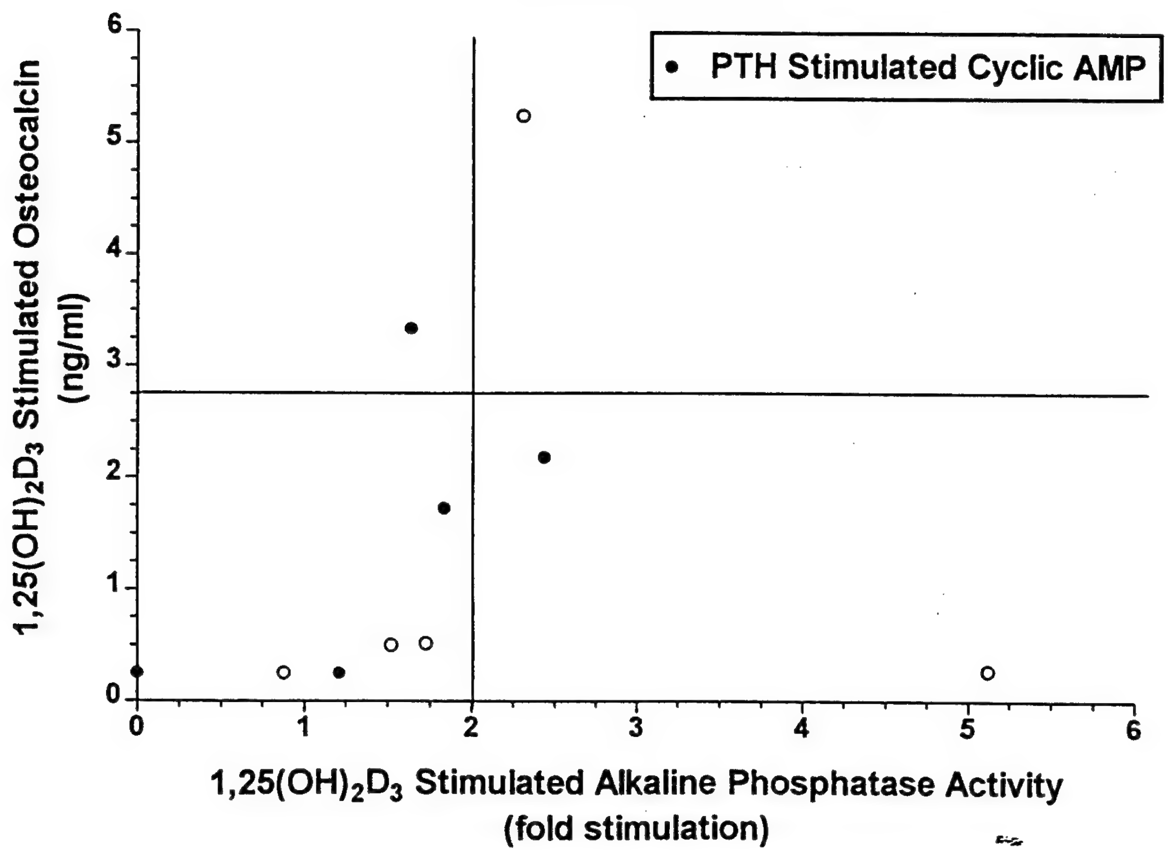
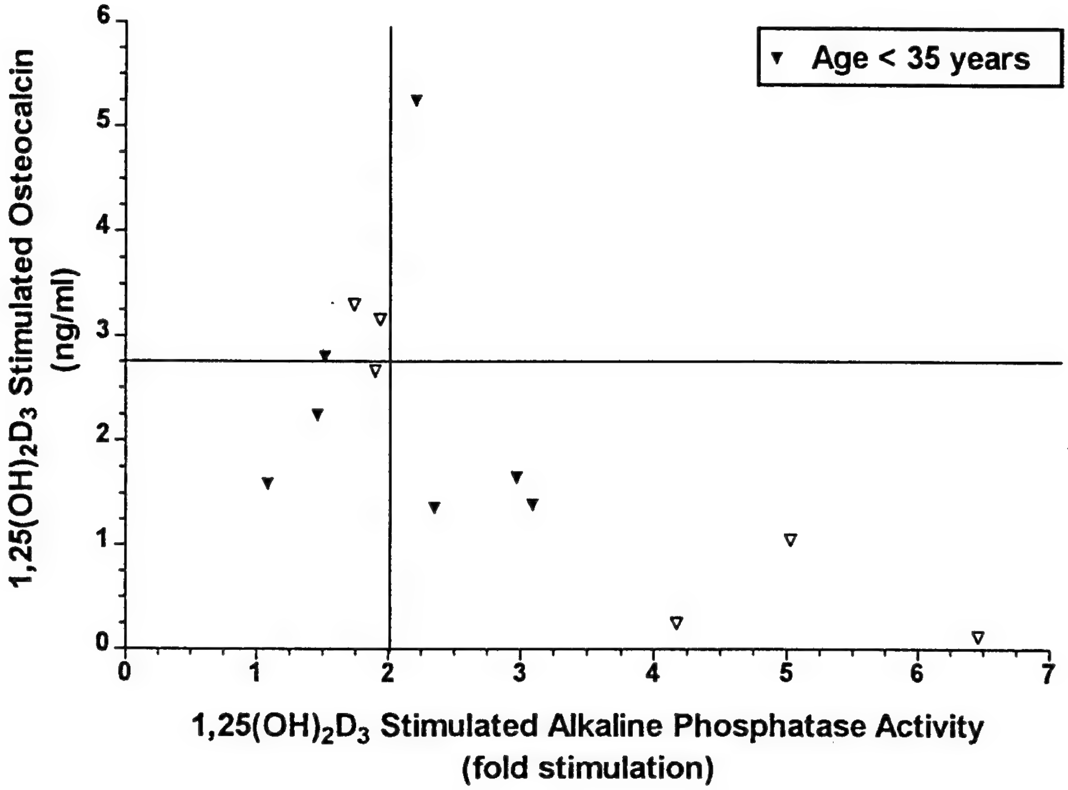


Figure 13. Age and Sex Verses $1,25(OH)_2D_3$ Stimulated Osteocalcin and Alkaline Phosphatase Activity in DMEM

Top. Scatter diagram plotting $1,25(OH)_2D_3$ stimulated/basal alkaline phosphatase activity verses $1,25(OH)_2D_3$ stimulated osteocalcin for cell populations explanted and maintained in DMEM. Arbitrary lines were drawn at points representing a 2 fold stimulation of alkaline phosphatase activity and 2.75 ng/ml of $1,25(OH)_2D_3$ stimulated osteocalcin production with cell populations which mineralized plotting in the upper left quadrant of the diagram. Age of individual donating PDL tissue for explant had no influence upon those cell populations which mineralized.

Bottom. Scatter diagram plotting $1,25(OH)_2D_3$ stimulated/basal alkaline phosphatase activity verses $1,25(OH)_2D_3$ stimulated osteocalcin for cell populations explanted and maintained in DMEM. Arbitrary lines were drawn at points representing a 2 fold stimulation of alkaline phosphatase activity and 2.75 ng/ml of $1,25(OH)_2D_3$ stimulated osteocalcin production with cell populations which mineralized plotting in the upper left quadrant of the diagram. Sex of individual donating PDL tissue for explant had no influence upon those cell populations which mineralized.

PDL Cells Explanted in DMEM



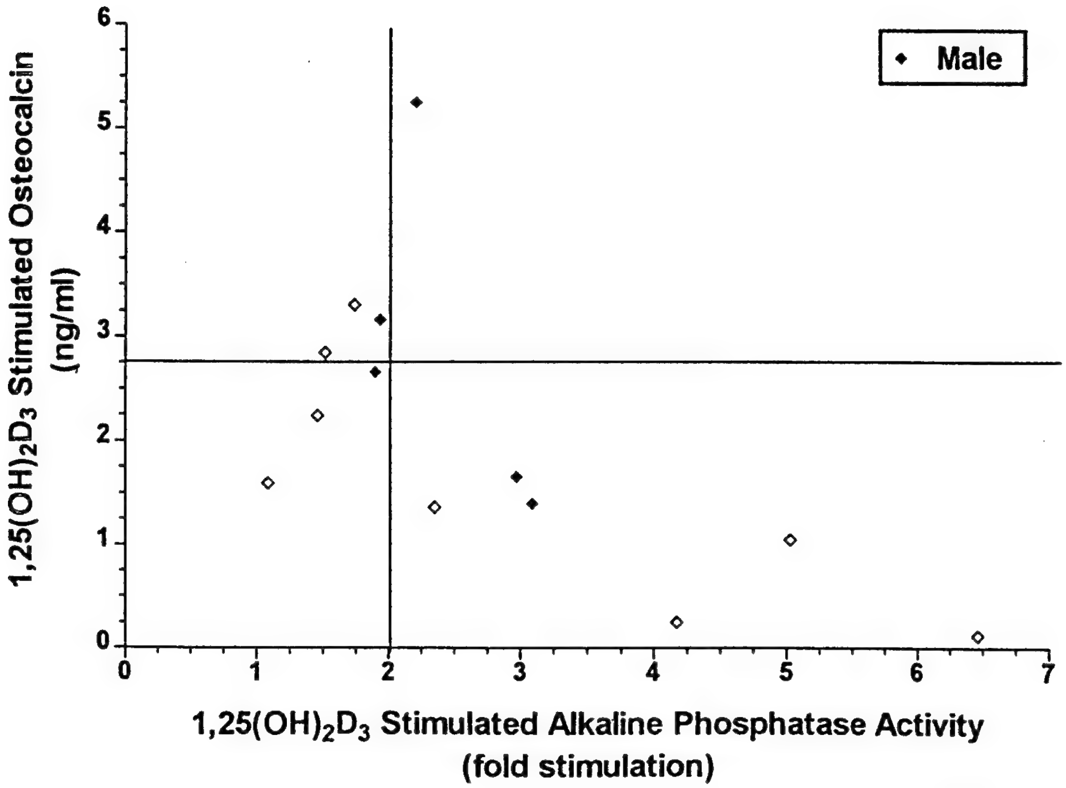
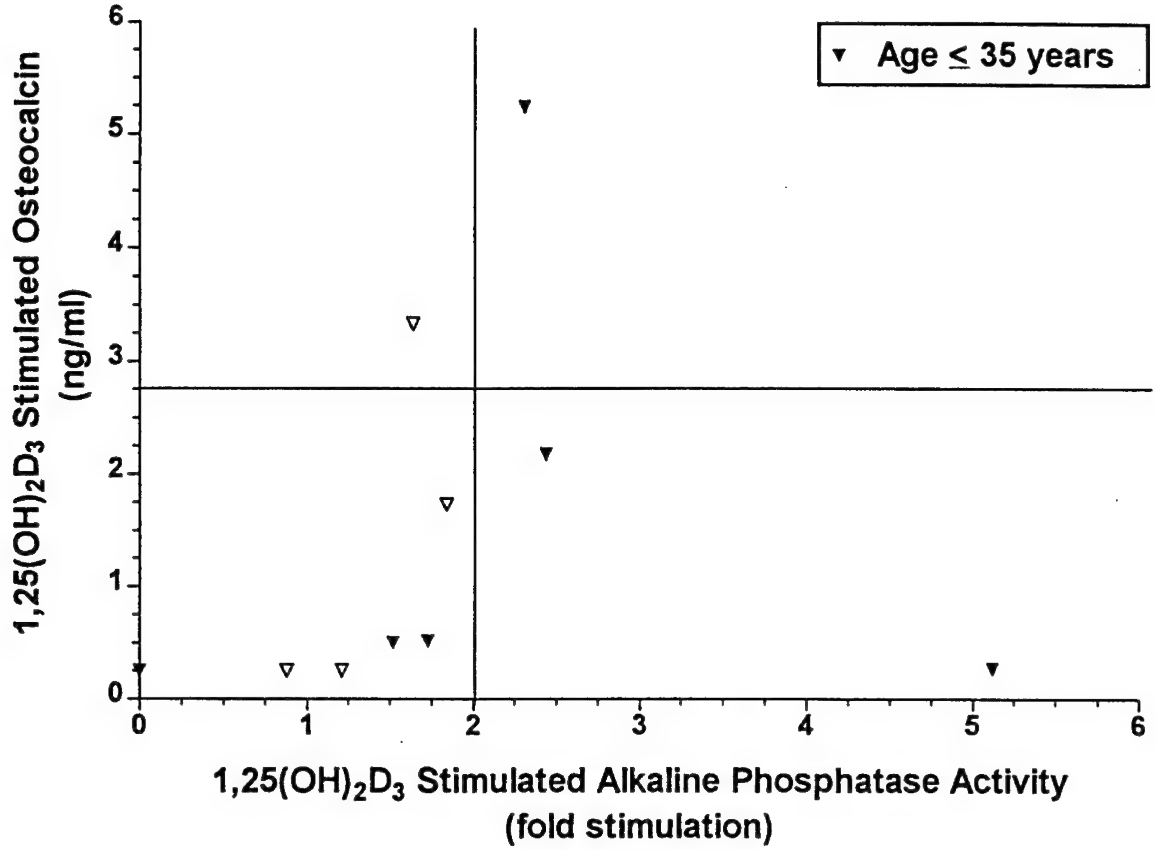


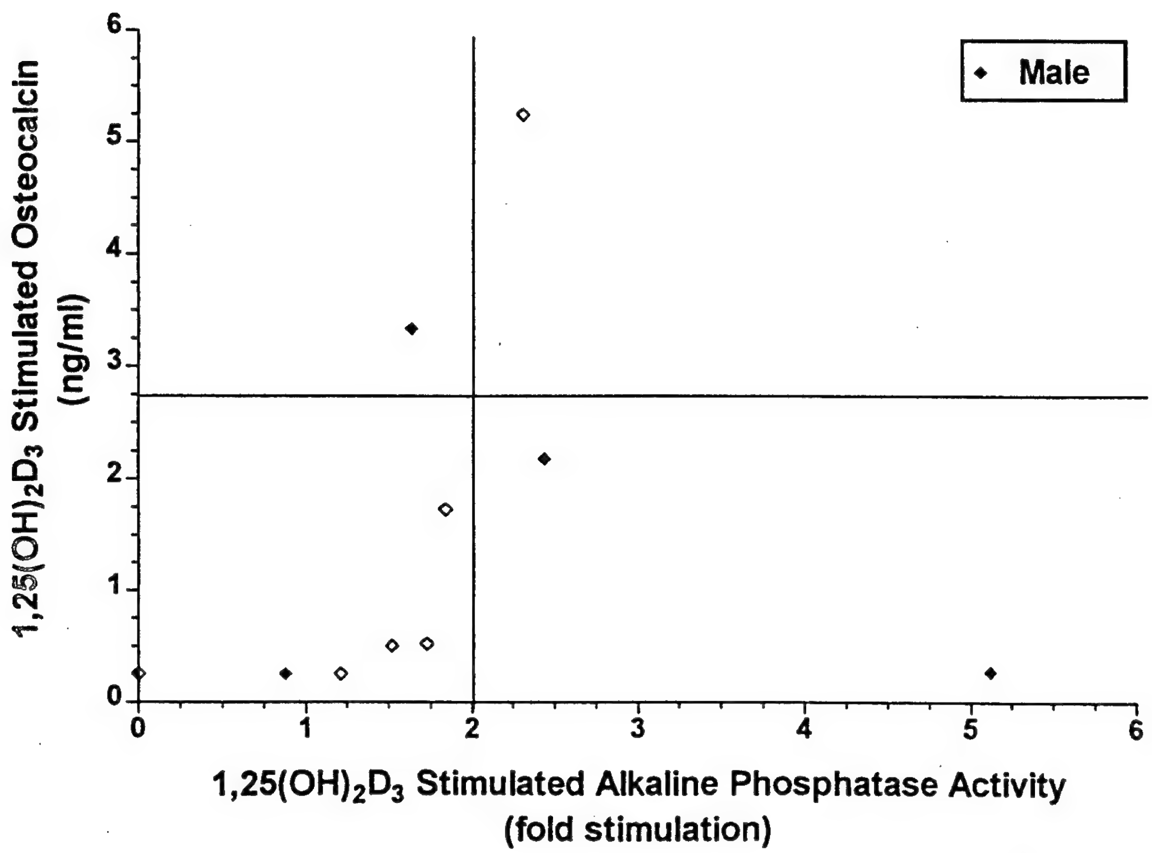
Figure 14. Age and Sex Verses 1,25$(OH)_2D_3$ Stimulated Osteocalcin and Alkaline Phosphatase Activity in Low Calcium Medium

Top. Scatter diagram plotting 1,25$(OH)_2D_3$-stimulated/basal alkaline phosphatase activity verses 1,25$(OH)_2D_3$-stimulated osteocalcin for cell populations explanted and maintained in low calcium medium. Arbitrary lines representing a 2 fold stimulation of alkaline phosphatase activity and 2.75 ng/ml of 1,25$(OH)_2D_3$-stimulated osteocalcin production were drawn on the plot with cell populations which mineralized plotting in the upper left quadrant of the diagram. Age of individual donating PDL tissue for explant did not correlate with those cell populations which mineralized or did not mineralize.

Bottom. Scatter diagram plotting 1,25$(OH)_2D_3$-stimulated/basal alkaline phosphatase activity verses 1,25$(OH)_2D_3$-stimulated osteocalcin for cell populations explanted and maintained in low calcium medium. Arbitrary lines representing a 2 fold stimulation of alkaline phosphatase activity and 2.75 ng/ml of 1,25$(OH)_2D_3$-stimulated osteocalcin production were drawn on the plot with cell populations which mineralized plotting in the upper left quadrant of the diagram. Sex of individual donating PDL tissue for explant did not correlate with those cell populations which mineralized or did not mineralize.

PDL Cells Explanted in Low Calcium Medium





the transformed gene is turned off (Jat and Sharp, 1989). In the present study, the temperature of 37°C was evaluated as the non-permissive temperature for evaluation of the previously transformed cell line HPDL. This cell line, supplied by Dr. Mary MacDougall, was derived from human PDL and transformed using procedures described in methods. The cell line has previously been characterized as having an osteoblast-like phenotype. Because the PDL used for explant was obtained by scraping the root surface, it is speculated that the cells have a cementoblast-like phenotype (personal communication with Dr. Mary MacDougall). HPDL cell populations have not been cloned and should, therefore, be considered heterogenous. The cell line was evaluated for basal and hormone stimulated alkaline phosphatase activity, osteocalcin and cAMP production, and the ability to proliferate at both the permissive and non-permissive temperatures (table 4). At both 33°C and 37°C, all HPDL cell populations had basal levels of alkaline phosphatase activity which were stimulated by VitD. Similarly, all cell populations had an increase production of osteocalcin in response to VitD. The only marker which was variable was cAMP production in response to PTH where 3 out of 5 cell populations had a significant response ($p < 0.05$). The cAMP response reported occurred at both the 33°C and 37°C temperature. The type of medium in which the cell populations were characterized did not influence the results of the characterization.

Results indicate that at the permissive temperature of 33°C, when the transformed gene is expressed, HPDL cell numbers increased in all three test media when evaluated over 500 hours (figure 15). A very different pattern of proliferation was observe when HPDL cells were evaluated at the non-permissive temperature of 37°C, when expression of the large T antigen is turned off. In both DMEM and low-calcium medium, there was an initial significant increase in

Table 4

CHARACTERIZATION OF HPDL CELL POPULATIONS AT 33^{0}C and 37^{0}C USING DULBECCO'S MODIFIED EAGLE'S MEDIUM AND LOW CALCIUM MEDIUM

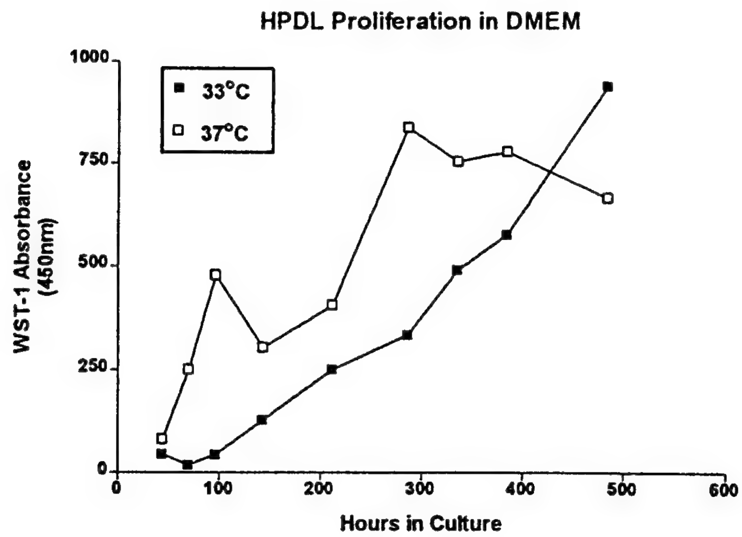
	Alkaline Phosphatase (pmol/ml)		Osteocalcin (ng/ml)		CAMP (pmol/ml)	
	VitD / Basal	Fold Stimulation	Basal	VitD	PTH / Basal	Fold Stimulation
HPDL-P8 DMEM 37^{0}C	344.5 / 119.8	2.9	BDL	2.11	4.7 / 4.8	0.98
HPDL-P13 DMEM 33^{0}C	349.3 / 61.4	5.7	BDL	1.54	13.79 / 11.43	1.21*
HPDL-P6 Low Ca^{++} 37^{0}C	317.7 / 145.1	2.2	BDL	1.44	3.88 / 2.35	1.65*
HPDL-P9 Low Ca^{++} 37^{0}C	251.4 / 214.8	1.2	BDL	2.92	4.42 / 4.46	0.99
HPDL-P12 Low Ca^{++} 33^{0}C	109.3 / 73.9	1.5	BDL	1.27	26.76 / 11.74	2.28*

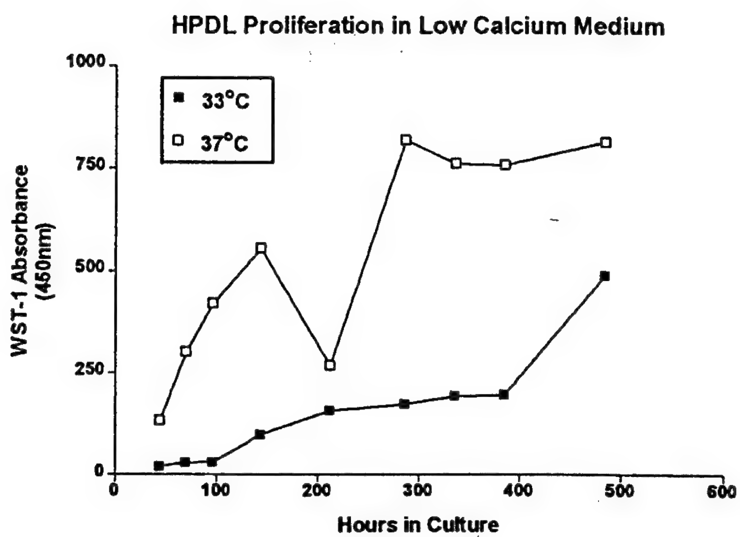
BDL = Below Detectable Limits

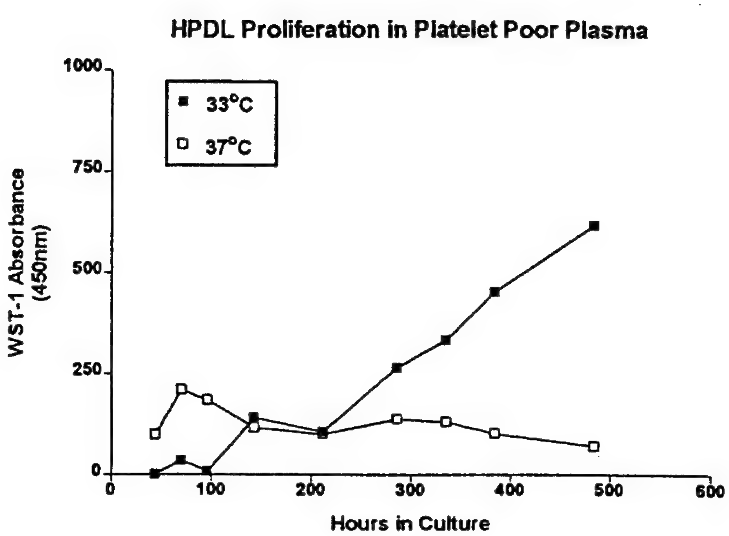
Cell population HPDL was characterized for the osteoblast-like phenotype following transformation using the SV-40 large T antigen. HPDL cell populations maintained in DMEM were characterized after passage 8 at the non-permissive temperature of 37^{0}C and after passage 13 at the permissive temperature of 33^{0}C. HPDL cell populations maintained in low-calcium medium were characterized after passage 6 and 9 at the non-permissive temperature and after passage 12 at the permissive temperature. All cell populations characterized responded to VitD stimulation with significant increases ($p < 0.05$) in alkaline phosphatase activity and osteocalcin production, while 3 of 5 cell populations had a significant cAMP response to PTH (*, $p < 0.05$).

Figure 15. Proliferation of Transformed Cell Population HPDL

Proliferation of cell population HPDL was evaluated at the permissive temperature of 33^0C, when the SV-40 T antigen was expressed, and the non-permissive temperature of 37^0C, when the transformed gene was turned off. Different test media consisting of DMEM (top graph), low calcium medium (middle graph) and platelet poor plasma medium (bottom graph) were used for evaluation at each temperature. Cells were plated at a lower density of 5000 cells/cm^2 for proliferation at 33^0C compared to 7,000 cells/cm^2 at 37^0C. Proliferation was measured using WST-1 absorbance (450 nm) at 9 different timepoints over 500 hours. Results show continual proliferation in all 3 test media over 500 hours at 33^0C. At 37^0C, initial proliferation plateaus in all 3 test media suggesting that the transformed gene has been shut down.


HPDL Proliferation in DMEM
33°C
37°C
WST-1 Absorbance (450nm)
Hours in Culture
0
250
500
750
1000
100
200
300
400
500
600


HPDL Proliferation in Low Calcium Medium
33°C
37°C
WST-1 Absorbance (450nm)
Hours in Culture
0
250
500
750
1000
100
200
300
400
500
600


HPDL Proliferation in Platelet Poor Plasma
33°C
37°C
WST-1 Absorbance (450nm)
Hours in Culture
0
250
500
750
1000
100
200
300
400
500
600

cell proliferation until 300 hours. Beyond this timepoint cell number began to decrease (figure 15). A similar pattern was observed for cells cultured in the PPP medium, except the decrease in cell numbers occurred earlier at 100 hours. HPDL cells were evaluated by phase contrast microscopy at 24 hours where differences in plating densities between 33^0C and 37^0C for all test media were noted (figures 16 and 17). Differences in cell morphology between cell populations cultured at 33^0C and 37^0C became apparent at the 212 hour timepoint (figures 18 and 19). At 33^0C, cells in all three test medium appeared spindle shaped with prominent nuclei and the presence of numerous mitotic figures. At 37^0C, cells in DMEM and low-calcium medium were larger and kite shaped and appeared to be undergoing degeneration. At 37^0C, cells grown in PPP were detached from the culture dish and presumed non-viable, although cell viability was not determined. These same morphological characteristics between HPDL cells expanded at 33^0C and 37^0C were noted at the 485 hour timepoint when compared to the 212 hour timepoint (figures 20 and 21).

2. <u>Transfections of PDL Cell Populations with SV-40 T Antigen</u>. Cells were selected for transformation using the SV-40 T antigen - temperature sensitive construct based on alkaline phosphatase activity after passage one. Fifteen cell populations were then infected with the SV-40 T antigen construct. Successful transformations occurred with cell populations 13-1 and 16-3. Figure 22 shows cell population 13-1 in the subconfluent and confluent states after initial explant before transformation. Similarly, figure 23 shows cell population 16-3 in the subconfluent and confluent states before transformation. Successful transformation was verified using immunohistochemistry to evaluate expression of the large T antigen in these cells. Figure 24 displays specific staining of the large T antigen in population 13-1 at both the permissive and non-

Figure 16. Photomicrographs of Cell Population HPDL Proliferation at 33^0C at 24 Hours

Photomicrographs depict HPDL cells plated at a density of 5,000 cells/cm^2 and expanded at the permissive temperature of 33^0C. Cell attachment and growth were noted at 24 hours in test media including DMEM (figure A), low calcium medium (figure B) and platelet poor plasma medium (figure C). All 3 media included addition of the neomycin analogue G418. No morphological differences were noted between media. Original magnification = 25X.

Figure 17. Photomicrographs of Cell Population HPDL Proliferation at 37^0C at 24 Hours

Photomicrographs depict HPDL cells plated at a density of 7,000 cells/cm^2 and expanded at the non-permissive temperature of 37^0C. Cell attachment and growth were noted at 24 hours in test media including DMEM (figure A), low calcium medium (figure B) and platelet poor plasma medium (figure C). No morphological differences were noted between media. Original magnification = 25X.

Figure 18. Photomicrographs of Cell Population HPDL Proliferation at 33^{0}C at 212 Hours

Photomicrographs depict HPDL cells expanded at the permissive temperature of 33^{0}C. Cell attachment and growth were noted at 212 hours in test media including DMEM (figure A), low calcium medium (figure B) and platelet poor plasma medium (figure C). All 3 media included addition of the neomycin analogue G418. No morphological differences were noted at the subconfluent state between cells growing in the different media. Original magnification = 25X.

Figure 19. Photomicrographs of Cell Population HPDL Proliferation at 37^0C at 212 Hours

Photomicrographs depict HPDL cells expanded at the non-permissive temperature of 37^0C. Cell attachment and growth was noted at 212 hours in test media including DMEM (figure A), low calcium medium (figure B) and platelet poor plasma medium (figure C). Cells expanded in platelet poor plasma exhibit an increased number of cells which have detached and appear round in shape. When compared with cells expanded at 33^0C, cells at the non-permissive temperature morphologically appear wider with increased cell detachment. Original magnification = 25X.

Figure 20. Photomicrographs of Cell Population HPDL Proliferation at 33^{0}C at 485 Hours

Photomicrographs depict HPDL cells expanded at the permissive temperature of 33^{0}C. Cell attachment and growth was noted at 485 hours in test media including DMEM (figure A), low calcium medium (figure B) and platelet poor plasma medium (figure C). All 3 media included addition of the neomycin analogue G418. No morphological differences were noted at the confluent state between media. Original magnification = 25X.

Figure 21. Photomicrographs of Cell Population HPDL Proliferation at 37^0C at 485 Hours

Photomicrographs depict HPDL cells expanded at the non-permissive temperature of 37^0C. Cell attachment and growth were noted at 485 hours in test media including DMEM (figure A), low calcium medium (figure B) and platelet poor plasma medium (figure C). All 3 media exhibit morphological differences that are consistent with cell degeneration when compared to cells expanded at 33^0C at the same timepoint. Increased cell detachment is also apparent. Original magnification = 25X.

Figure 22. Photomicrographs of Periodontal Ligament Explant 13-1

Figure A. Subconfluent cells derived from explanted PDL tissue are displayed 10 days after initiation of the explant culture. Original magnification = 25X.

Figure B. Confluent monolayer of cells derived from explanted PDL tissue just prior to first passage at 21 days. Original magnification = 25X

Figure 23. Photomicrographs of Periodontal Ligament Explant 16-3

Figure A. Subconfluent cells derived from explanted PDL tissue are displayed 10 days after initiation of the explant culture. Original magnification = 25X.

Figure B. Confluent monolayer of cells derived from explanted PDL tissue just prior to first passage at 21 days. Original magnification = 25X

Figure 24. Immunohistochemical Staining for SV-40 Large T Antigen

Figure A. Immunohistochemical staining of cell population 13-1 at the permissive temperature of 33^0C using antibody directed towards the large T antigen. Heavy staining of cell nuclei and light staining of cytoplasm and cell membranes can be noted. Original magnification = 25X.

Figure B. Immunohistochemical staining of cell population 13-1 at the non-permissive temperature of 37^0C using antibody directed towards the large T antigen. Significant decrease in staining is evident when compared to staining at 33^0C in figure A. Original magnification = 25X.

Figure C. Negative control of cell population 13-1 at the permissive temperature of 33^0C. Absence of staining is comparable to antibody staining at 37^0C seen in figure B. Original magnification = 25X

permissive temperatures. This staining is also representative of cell population 16-3. Significant staining is evident at the permissive temperature indicating that this construct has been successfully integrated in these cells. The dramatic reduction in staining at the non-permissive temperature indicates that expression of large T antigen is turned off. It was of interest to evaluate the phenotypes of these cells after transformation at both the permissive and non-permissive temperatures. Figure 25 shows cell populations 13-1 and 16-3 following transformation with the SV-40 T antigen.

Prior to transformation, both cell populations were evaluated biochemically for alkaline phosphatase activity following first passage. In the presence of VitD, there was a 1.8 fold increase in alkaline phosphatase activity in cell population 13-1, and a 1.2 fold increase in cell population 16-3 over basal activity (table 5). Cell numbers were increased in both of these cell populations until fourth passage was reached. At this time the cells were characterized again, and parallel cultures were plated to begin the transformation. In the presence of VitD biochemical alkaline phosphatase activity was increased 1.5 fold in cell population 13-1 and 2.4 fold in cell population 16-3 (table 6). The increase in VitD stimulation in population 16-3 from first passage to fourth passage is evidence of the heterogeneity of these PDL cell populations. Results of the histochemical evaluation of both basal and VitD-stimulated alkaline phosphatase were consistent with the biochemical analysis for both cell populations (figures 26 and 27). In addition, both cell population 13-1 and 16-3 responded to VitD stimulation with an increase in osteocalcin production, while only population 16-3 responded to PTH with an increase in cAMP production (table 7). Neither cell population showed evidence of mineralization.

Following infection of 13-1 and 16-3 with the SV-40 T antigen, the transformed cell

Figure 25. Photomicrographs of Transformed Cell Populations 13-1 and 16-3

Figure A. Photomicrograph of cell population 13-1 following transformation using SV-40 T antigen. Cell morphology is similar to the non-transformed state. Original magnification = 25X.

Figure B. Photomicrograph of cell population 16-3 following transformation using SV-40 T antigen. A change in cell morphology from spindle shaped seen before transformation to a more round, octagonal shape following transformation can be noted. Original magnification = 25X.

Table 5

ALKALINE PHOSPHATASE ACTIVITY OF CELL POPULATIONS 13-1 AND 16-3 FOLLOWING PASSAGE ONE PRIOR TO TRANSFORMATION

	Basal (pmol/ml)	VitD (pmol/ml)	Fold Stimulation
13-1P1	761.5	1371.5	1.8
16-3P1	2357.5	2855.5	1.2

Cell populations 13-1 and 16-3 were evaluated for basal and VitD-stimulated alkaline phosphatase activity following passage one. The responses are significant with $p < 0.05$.

Table 6

CHARACTERIZATION OF CELL POPULATIONS 13-1 AND 16-3 FOLLOWING PASSAGE FOUR PRIOR TO TRANSFORMATION

	Alkaline Phosphatase (pmol/ml)		Osteocalcin (ng/ml)		CAMP (pmol/ml)	
	VitD / Basal	Fold Stimulation	Basal	VitD	PTH / Basal	Fold Stimulation
13-1P4	942.5 / 619.9	1.5*	BDL	0.51	3.3 / 3.6	0.92#
16-3P4	816.3 / 335.2	2.4*	BDL	2.18	5.6 / 3.5	1.6#

BDL = Below Detectable Limits

Cell populations 13-1 and 16-3 were characterized for the osteoblast-like phenotype before transformation. Characterization was performed following fourth passage. *, # indicate significantly different ($p < 0.05$) from respective basal level.

Figure 26. Photomicrographs of Histochemical Alkaline Phosphatase Activity of Cell Population 13-1

Figure A. Photomicrograph of basal levels of alkaline phosphatase activity in cell population 13-1. Substrate for the alkaline phosphatase reaction was fast violet B salt. Original magnification = 25X.

Figure B. Photomicrograph of $1,25(OH)_2D_3$-stimulated alkaline phosphatase activity in cell population 13-1. Substrate for the alkaline phosphatase reaction was fast violet B salt. Original magnification = 25X.

Figure 27. Photomicrographs of Histochemical Alkaline Phosphatase Activity of Cell Population 16-3

Figure A. Photomicrograph of basal levels of alkaline phosphatase activity in cell population 16-3. Substrate for the alkaline phosphatase reaction was fast violet B salt. Original magnification = 25X.

Figure B. Photomicrograph of $1,25(OH)_2D_3$-stimulated alkaline phosphatase activity in cell population 16-3. Substrate for the alkaline phosphatase reaction was fast violet B salt. Original magnification = 25X.

Table 7

CHARACTERIZATION OF CELL POPULATIONS 13-1 AND 16-3 FOLLOWING TRANSFORMATION

	Alkaline Phosphatase (pmol/ml)		Osteocalcin (ng/ml)		cAMP (pmol/ml)	
	VitD / Basal	Fold Stimulation	Basal	VitD	PTH / Basal	Fold Stimulation
13-1P9 33^0C	119.2 / 37.3	3.2*	BDL	1.56	3.6 / 3.6	1.0
13-1P12 37^0C	272.2 / 74.1	3.7*	BDL	4.17	7.4 / 9.7	0.76#
16-3P6 33^0C	BDL		BDL	BDL	3.5 / 3.9	0.9

BDL = Below Detectable Limits

Cell populations 13-1 and 16-3 were characterized for the osteoblast-like phenotype following transformation using the SV-40 large T antigen. Cell population 13-1 was characterized at the permissive temperature of 33^0C following passage 9 and the non-permissive temperature of 37^0C following passage 12. Cell population 16-3 was characterized after passage 6 at the permissive temperature of 33^0C. *, # indicate significantly different ($p < 0.05$) from respective basal level.

populations were expanded at the permissive temperature of 33^{0}C. Cell population 13-1 was characterized at passage 9 and 16-3 was characterized at passage 6 after transformation. Cell population 13-1 retained the phenotypic characteristics present before transformation (passage 4) including increases in alkaline phosphatase activity and osteocalcin production over basal levels following stimulation with VitD. Furthermore, cell population 13-1 exhibited these characteristics at the non-permissive temperature of 37^{0}C at passage 12 when expression of the T antigen was turned off. Unlike cell population 13-1, cell population 16-3 did not retain the phenotypical characteristics present before transformation (passage 4). Basal levels of alkaline phosphatase activity and osteocalcin production were decreased or non-detectable and did not respond to stimulation with VitD. Similarly, the cAMP response to PTH was absent following transformation. Thus, following transformation cell population 13-1 continued to display phenotypic characteristics consistent with the osteoblast-like phenotype, while cell population 16-3 displayed phenotypic characteristics associated with fibroblast-like cells. To date, both cell populations have been passaged over 30 times and may be considered a stable cell line. Furthermore, stock cultures of both cell populations have been frozen in liquid N_2 for long term storage and have been used to successfully establish new cultures when brought out of the frozen state.

C. Growth in Semisolid Medium

1. Evaluation of Anchorage Independent Growth. Selection of PDL cells with an osteoblast-like phenotype was also attempted using the soft agar transformation assay. Ten cell populations were dispersed in the soft agar assay for evaluation of anchorage independent growth. No large colony formation (90-100 μm; Guenther *et al.*, 1989) was noted in any of the soft agar

assays evaluated. Therefore, the largest colonies estimated to be 50-60 μm when viewed by phase contrast microscopy were collected for culture in traditional cell culture wells (figure 28). Most of these colonies were unable to attach and survive when returned to monolayer culture. However, one cell colony, 14-4, was carried through seven passages in order to achieve the number of cells required for characterization.

2. Characterization. First passage cells from cell population 14-4 were used in the soft agar assay prior to any characterization. Cell population 14-4 was characterized at passage four before acquisition of anchorage independent growth in soft agar (table 8) and displayed a 5.1 fold increase in alkaline phosphatase activity following stimulation by VitD. Histochemical evaluation of alkaline phosphatase activity was consistent with these biochemical results (figure 29). Neither basal or VitD stimulated osteocalcin production was present. Both a cAMP response to PTH and evidence of mineralization were absent as well. Cell population 14-4 was characterized at passage seven following the soft agar assay (table 8). The increase in alkaline phosphatase activity in response to VitD decreased to only 1.3 fold. Osteocalcin production remained non-detectable. However, the cells now responded to PTH with a 7 fold stimulation in cAMP. Again no mineralization was detected.

3. Transformation. Interestingly, after the sixth passage of cell population 14-4 following the soft agar assay, there appeared clumps of cells with a strikingly different morphology (figure 30). By contrast with the spindle shaped cells which dominated in the culture, these newly appearing cells were octagonal or round. When the cultures were evaluated histochemically for alkaline phosphatase activity, these newly appearing cells stained heavily for the enzyme while staining was low or absent over the spindle shaped cells (figure 31). As cell

Figure 28. Photomicrographs of Soft Agar Colony Formation of Cell Population 14-4

Figure A. Photomicrograph of cell population 14-4 after six weeks in soft agar. Colony formation is evident with 3 colonies presenting with increased size measuring between 50-60 μm. Original magnification = 10X.

Figure B. Photomicrograph of cell colony which has been extracted from soft agar depicted in figure A. The cell colony has been placed in a 96 well cell culture plate for attachment and expansion utilizing DMEM with 10% FBS. Original magnification 50X.

Table 8

CHARACTERIZATION OF CELL POPULATION 14-4 BEFORE AND AFTER SPONTANEOUS TRANSFORMATION

	Alkaline Phosphatase (pmol/ml)		Osteocalcin (ng/ml)		CAMP (pmol/ml)	
	VitD / Basal	Fold Stimulation	Basal	VitD	PTH / Basal	Fold Stimulation
14-4P4	343.7 / 67.5	5.1*	BDL	2.7	0.5 / 1.2	0.42
14-4P7 after soft agar assay	802.7 / 638.3	1.3*	BDL	BDL	1.6 / 0.2	7.2#

BDL = Below Detectable Limits

Cell population 14-4 was characterized for the osteoblast-like phenotype before transformation at passage four and after transformation at passage seven. *, # indicate significantly different ($p < 0.05$) from respective basal levels.

Figure 29. Photomicrographs of Histochemical Alkaline Phosphatase Activity of Cell Population 14-4 Prior to Soft Agar Assay

Figure A. Photomicrograph of basal levels of alkaline phosphatase activity in cell population 14-4 at passage four before soft agar experimentation. Substrate for the alkaline phosphatase reaction was fast violet B salt. Original magnification = 25X.

Figure B. Photomicrograph of $1,25(OH)_2D_3$-stimulated alkaline phosphatase activity in cell population 14-4 at passage four before soft agar experimentation. Substrate for the alkaline phosphatase reaction was fast violet B salt. Original magnification = 25X.

Figure 30. Photomicrographs of Transforming Cell Population 14-4

Figure A. Photomicrograph of cell population 14-4 established from colony growth using soft agar experimentation. The cell population is depicted at passage 6 with cells exhibiting two morphological shapes. Cells with a round, octagonal shape began forming clusters within a previously homogenous population of spindle-shaped cells. With further passage, the round, octagonal shaped cells overgrew the cell culture to form a homogenous population of cells with this morphological shape. Original magnification = 25X.

Figure B. Higher power view of figure A depicting the round, octagonal shaped cells clustering within cell population 14-4 which was established from a single colony extracted from soft agar. Original magnification 50X.

Figure 31. Photomicrographs of Histochemical Alkaline Phosphatase Activity of Transforming Cell Population 14-4

Figure A. Photomicrograph of cell population 14-4 established from colony growth using soft agar experimentation. Histochemical staining for basal alkaline phosphatase activity is depicted at passage seven. Cells which present morphologically as spindle-shaped show evidence of sparse staining for alkaline phosphatase while cells with a round, octagonal shape stain heavily for the enzyme. Substrate for the alkaline phosphatase reaction was fast blue BB salt. Original magnification = 25X.

Figure B. Higher power view of figure A depicting abundant histochemical alkaline phosphatase staining of round, octagonal shaped cells found at passage 7 in cell population 14-4. Substrate for the alkaline phosphatase reaction was fast blue BB salt. Original magnification 50X.

population 14-4 was expanded and passaged, these alkaline phosphatase positive cells increased in number until a relatively pure culture of cells with this morphology were obtained. Furthermore, this cell population has now been passaged over 50 times and these cells remain the dominant population of cells. Because this cell population was derived from a cell colony originated from a single cell in the soft agar assay, it may be considered clonal. The appearance of the cells with new morphology and the continued proliferation without senescence for over 50 passages, indicates that this cell population has undergone spontaneous transformation and is now an immortal cell line. Negative staining for the presence of the SV-40 T antigen in these cells confirmed that there was no cross infection from the other transformed cell populations evaluated. This transformed cell population has also been frozen and successfully brought out of liquid N_2 storage to establish new cultures.

IV. DISCUSSION

A. Cell Populations

A total of 29 cell populations derived from 110 human PDL explant cultures were successfully established in at least one of three culture media and expanded for characterization in this study. Therefore, viable explant cultures, based on the ability of PDL cell populations to proliferate in culture, were 26.4% of the cultures originally initiated. This percentage is higher than that reported by Piche *et al.*, (1989a) where only 10% of the explant cultures initiated resulted in the establishment of successful PDL cell populations. This difference may be due to variations in the explant culture techniques utilized in the two studies, as well as to the methods used for obtaining the PDL tissue. By contrast with the study of Piche *et al.*, (1989a), tissue in the present study was atraumatically removed from the root without scraping the surface. As a result of gentle removal of PDL tissue, the presence of cementum-derived cells may be avoided in the otherwise heterogenous cell populations initially explanted. Mature cementum-derived cells likely represent a differentiated cell population with a limited ability to proliferate. Piche *et al.*, (1989a) observed explant cultures for outgrowth of cells for only 3-4 weeks prior to terminating them as unsuccessful. Since their cell populations were derived from PDL tissue vigorously scraped from the root surface, they may have been enriched with cells with the capacity to mineralize due to the presence of cementum-derived cells. Therefore, many of their cultures may have been terminated before appreciable proliferation of cells was observed, accounting for the decreased percentage of viable cell populations reported in their study. In support of this explanation is the fact that cell populations which mineralized in the present study averaged

greater than 30 days in culture before first passage. Thus, how and where PDL tissue is first obtained for initiation of explant cultures is likely to influence the phenotypic characteristics expressed, and underscores the heterogeneity of cell populations resident in the PDL.

Of the three media used in this study, DMEM containing 5% PPP proved to be the least useful for successfully establishing and maintaining cell populations from the PDL. The percentage of cell populations reaching fourth passage with enough cells for characterization was markedly lower in the PPP containing media compared to cell populations expanded in DMEM and low-calcium medium. Furthermore, the time to reach first passage after initiation of the explants was significantly greater for those cell populations that were successfully explanted, maintained and expanded in the PPP containing medium compared to the other two media used. The differences in the success rates between the various media may be explained by the decreased presence of growth factors in PPP medium compared to media containing fetal bovine serum. Cell proliferation in PPP medium is dependent on endogenous growth factor production by the cells themselves, particularly platelet derived growth factor (PDGF). It has been reported that both bone cell populations and osteoblast-like cell populations derived from PDL tissue are able to proliferate in the absence of PDGF, while fibroblasts and fibroblast-like cell populations from PDL are not. It is now known that the osteoblast is able to produce PDGF while fibroblasts are dependent on exogenous PDGF to support their proliferation (Piche *et al.*, 1989a; Piche *et al.*, 1989b). Therefore, the poor results reported in this study regarding the number of cell populations that expanded and characterized in PPP medium were both disappointing and unexpected. In the absence of unidentified technical problems, the results indicate that osteoblast-like populations were extremely rare in explanted PDL tissue. If this is true, then cells with a

phenotype consistent with that of mineral producing cells are likely tightly adherent to the root surface or closer to the PDL-bone interface and not generally distributed in the soft tissue portions of the PDL. The fact that only 4 cell populations with the ability to mineralize were established in the fetal bovine serum containing media supports this concept.

B. Characterization of PDL Cell Populations

Numerous studies evaluating the phenotypic characteristics of PDL cells have consistently reported expression of basal and $1,25(OH)_2D_3$-stimulated alkaline phosphatase activity (Kawase *et al.*, 1988; Somerman *et al.*, 1988; Piche *et al.*, 1989a; Nojima *et al.*, 1990; Ogata *et al.*, 1995; Goseki *et al.*, 1995; Giannopoulou and Cimasoni, 1996; Carnes *et al.*, 1997; Liu *et al.*, 1997). High basal alkaline phosphatase activity is characteristic of osteoblasts while fibroblasts routinely express only very low levels of this enzyme. In addition, alkaline phosphatase activity is markedly increased in osteoblasts following stimulation by $1,25(OH)_2D_3$, while this hormone has no effect on the activity in fibroblasts. Those cell populations were termed osteoblast-like and there has been great interest in identifying their potential for involvement in the maintenance and regeneration of the periodontal ligament. Identification and further characterization of alkaline phosphatase positive cell populations supports the concept that the PDL contains cell populations capable of serving as progenitors for each of the component tissues, including cementum and bone. In this study 25 out of 28 cell populations responded to stimulation by $1,25(OH)_2D_3$ with an increase in alkaline phosphatase activity. Histochemical evaluation for alkaline phosphatase activity was consistent with results obtained biochemically. Stimulation with $1,25(OH)_2D_3$ resulted in a uniform increase in the number of cells staining positive for alkaline phosphatase activity, rather than an increased intensity of staining in those cells expressing the enzyme under

basal conditions. This provides evidence that the cell population, as a whole, is responding to the hormone rather than a select subpopulation of cells. Alkaline phosphatase is expressed by the osteoblast early in the differentiation stage prior to the onset of mineralization. Expression of this enzyme decreases as the osteoblast produces a mature matrix, which ultimately mineralizes (Lian and Stein, 1992). Most cell populations established in the study expressed both basal and $1,25(OH)_2D_3$-stimulated alkaline phosphatase activity suggesting that they may have begun to differentiate along an osteogenic pathway, while only a very few cell populations are fibroblast-like based on the alkaline phosphatase data. Since alkaline phosphatase expression is most pronounced before mineralization, other markers for the mature osteoblast-like phenotype are needed to further characterize these cell populations.

The hallmark of a differentiated osteoblast in cell culture is production of a mineralized matrix. In addition to $1,25(OH)_2D_3$-responsive alkaline phosphatase, cells which are able to mineralize in culture also respond to this hormone with an increase in osteocalcin production. Thus, these three markers were utilized as phenotypical markers of an osteoblast-like phenotype in order to further characterize the established PDL cell populations. Of the 29 cell populations evaluated, 4 cell populations were found to mineralize in the presence of 2.5 mM β-glycerophosphate. When evaluated on a scatter diagram, it was determined that those cell populations that mineralized, responded to $1,25(OH)_2D_3$ with increases in osteocalcin production greater than 2.75 ng/ml while the increases in $1,25(OH)_2D_3$-stimulated alkaline phosphatase activity were 2 fold or less. Thus, most all of the PDL cell populations established responded to stimulation by $1,25(OH)_2D_3$ with increases in alkaline phosphatase activity, the increases in those that mineralized being less than 2-fold. The characteristics observed for the PDL cell populations

which mineralized are consistent with the model proposed by Lian and Stein (1992) for osteoblast growth and differentiation. During the stage of mineralization, osteocalcin is maximally expressed while the expression of alkaline phosphatase activity is decreased compared to the expression during the extracellular matrix maturation stage. Using these same parameters, other markers for the osteoblast phenotype were evaluated. In those cell populations which mineralized, 4 of 4 cell populations responded to stimulation by PTH with decreases in alkaline phosphatase activity while 3 of 4 increased cAMP production in response to the hormone. It is unknown why the fourth population did not also present with increased PTH-stimulated cAMP production. When these same osteoblast-like phenotypic markers were evaluated in the PDL cell populations, which did not mineralize in this study, considerable variability was apparent. Again, it is suggested that the other PDL cell populations established may represent cells in the osteoblast-like pathway, but are less differentiated when compared to those cell populations that mineralized in culture. These cells may represent precursors of the more differentiated cell type requiring appropriate stimulation to continue development of the mature phenotype. This variability in response may also be explained by the heterogeneity reported in PDL cell populations, and these cell populations may not be at all related to mineral forming cells. More experimentation will be required to answer this question.

Mineralization of PDL cell populations in the present study was evaluated using the stringent conditions proposed by Boskey *et al.*, (1992). These conditions were selected because they have been consistently reported to produce a calcified matrix closely resembling that *in vivo*, with an absence of artifactual, non-physiologic mineral deposition. Optimal conditions utilized 2.5 mM β-glycerophosphate as a source of organic phosphate. Thus, the concentration of β-

glycerophosphate in the present study is lower than the concentration used in most other studies evaluating mineralization in PDL cell cultures (Arceo *et al.*, 1991; Cho *et al.*, 1992; Mukai *et al.*, 1993; Nohutcu *et al.*, 1997; Carnes *et al.*, 1997; Liu *et al.*, 1997). Experiments to optimize the mineralizing conditions were not carried out in the present study, and the conditions employed may have been too stringent for evaluation of mineralization in PDL cell populations. Additional cell populations that had similar levels of $1,25(OH)_2D_3$-stimulated osteocalcin production and alkaline phosphatase activity (5-2P4 and 9-1P6) to the 4 populations which mineralized, may also have mineralized if conditions had been optimal. Thus, it is possible that the number of populations expressing the mature osteoblast-like phenotype may be underestimated in the present study.

In order to ascertain if any parameters of the patient population influenced mineralization in PDL cell cultures, the effect of patient age and sex was evaluated. The effect of sex of the donor on expression of PDL cell phenotype has not been reported, and the effects of age on cell proliferation have only been reported in abstract form (Hoang *et al.*, 1997b). Limited information available from the present study indicates that age and sex of donor PDL tissue did not influence expression of a mineralizing phenotype.

C. Selection of the Osteoblast-Like Phenotype by Selective Culture Methods

One aim of the present study was to evaluate the ability of low-calcium and PPP containing medium, when compared to standard culture medium, to select for the osteoblast-like phenotype from heterogenous PDL cell populations. Four of 24 cell populations established in either DMEM or low-calcium media mineralized in culture, while none of the cell populations established in PPP medium expressed this phenotype characteristic of mature mineralizing cells.

These results indicate that regardless of the type of media used, only a small percentage of the cultures established presented with a fully differentiated phenotype of the mature osteoblast. As discussed previously, the method of PDL tissue collection may have influenced the results of the present study. Cell populations expressing a mature osteoblast-like phenotype likely remained adherent to the root surface, while less differentiated or unrelated cell populations were removed with the soft PDL tissue. Since most of the cell populations established expressed markers characteristic of the osteoblast-like phenotype but did not mineralize, the data are consistent with the suggestion that cultures established in all three media represent cell populations with varying characteristics consistent with a less differentiated osteoblast-like phenotype. When cell populations established in DMEM and low-calcium containing medium were compared on the basis of $1,25(OH)_2D_3$-stimulated alkaline phosphatase activity or osteocalcin production, no differences were appreciated in the populations. This indicates that neither culture media provided conditions that favored proliferation and expansion of the particular phenotypes identified in this study. Thus, although phenotypic heterogeneity in the PDL was evident without clear selection for the mature osteoblast phenotype, the cell populations established may represent precursors of this more fully differentiated phenotype.

Selection for an osteoblast-like phenotype through anchorage independent growth was evaluated in a clonal soft agar assay. As reported by Guenther *et al.*, (1989) using postnatal rat calvaria cells, bone cells with characteristics of the osteoblast phenotype can be induced by TGF-β to assume reversibly the transformed phenotype. Transformation of osteoblasts reported by Guenther *et al.*, (1989) occurred in large (90-100 μm) colonies formed in soft agar. These large colonies were selected, placed back into monolayer culture, and expanded by anchorage

dependent growth. In the present study, although multiple colonies of increased size were formed, none reached the 90-100 μm size reported in the Guenther *et al.*, (1989) study. The largest cell colonies that formed (50-60 μm) were, however, selected for culture using anchorage dependent conditions. With one exception, those colonies failed to proliferate when returned to monolayer culture. One cell colony, designated 14-4, did proliferate and was expanded for characterization. Cell population 14-4 was one of the larger colonies formed, and thus, cell colony size may be a key indicator of successful transformation of the osteoblast-like phenotype from the PDL in this assay as well. Geunther *et al.*, (1989) also used media supplemented with 20% bone conditioned media during expansion. Since no comparable substitute for this addition was available, expansion of selected colonies proceeded without this addition. This may have been a factor in the inability to obtain multiple cell populations using this technique. When characterized, cell population 14-4 responded to stimulation with $1,25(OH)_2D_3$ with increased alkaline phosphatase activity and to PTH-stimulated cAMP production. Mineralization and osteocalcin production were absent, and it is concluded that cell population 14-4 displayed phenotypic characteristics consistent with a less differentiated osteoblast-like cell population.

D. Transformation Using SV-40 Large T Antigen

In order to obtain stable cell lines for future *in vitro* investigation, PDL cell populations were transformed by retroviral infection using the SV-40 large T antigen with a temperature sensitive construct and the neomycin resistance gene. This temperature sensitive characteristic of transformed cell populations using the SV-40 T antigen becomes especially important when evaluating cell populations for the osteoblast-like phenotype. Using the model of osteoblast growth and differentiation proposed by Lian and Stein (1992), three stages consisting of

proliferation, extracellular matrix maturation and mineralization are present for normal diploid cells of the bone cell phenotype. Transformed cell populations lose the transition periods identified for these three stages. Therefore, long term studies utilizing transformed cell lines may not be consistent with those found in non-transformed cell populations. The ability to turn off the transformed gene and study osteoblast cell populations under conditions resembling primary conditions will be of great benefit.

The ability to turn off the transformation gene by placing the cell populations at a higher non-permissive temperature was evaluated using a previously transformed cell population designated HPDL. This cell line is a heterogenous PDL cell population obtained by methods similar to those used in the present study. Previous characterization of the HPDL cell population has revealed that it possesses characteristics of an osteoblast-like phenotype including the ability to mineralize in culture (personal communication with Dr. Mary MacDougall). Proliferation of HPDL was evaluated at the permissive temperature of 33^{0}C and the non-permissive temperature of 37^{0}C. Results indicate that when cell population HPDL is placed at the higher temperature, cell proliferation decreases over time when compared to proliferation at 33^{0}C. The results were consistent when proliferation was evaluated in all three culture media used in this study. Furthermore, as the cell populations reached the end of the evaluation period at 37^{0}C, they began to deteriorate and presented morphologically as non-proliferative cells with evidence of degenerative changes. This is consistent with the characterized phenotype of the HPDL cell population in non-transformed culture, as that of a fully differentiated, post-mitotic cell. Immunohistochemical staining for the T antigen was positive at 33^{0}C. A significant decrease in staining, similar to a negative control, was evident at the higher temperature of 37^{0}C. Thus, the

ability of the temperature sensitive construct to turn off expression of the transformation gene was verified. This indicates that cell populations transformed using this method, when place at a temperature of 37^{0}C, may be studied under conditions resembling non-transformed cells.

HPDL cell populations were also characterized at the permissive and non-permissive temperatures without a loss of the characterized phenotype. Characterization consistently identified this cell population as having basal and $1,25(OH)_2D_3$-stimulated levels of alkaline phosphatase activity and $1,25(OH)_2D_3$-modulated osteocalcin production. These results were consistent with previous characterization performed on this cell population (personnel communication with Dr. Mary MacDougall). The HPDL cell populations were not evaluated for mineralization in the present study

Two cell populations identified as 13-1 and 16-3 were established in the present study and subsequently transformed using the SV-40 large T antigen. These cell populations were chosen based on the increase in alkaline phosphatase activity in response to $1,25(OH)_2D_3$-stimulation after first passage. These same populations were expanded and characterized at fourth passage when the cultures continued to express a phenotype characteristic of osteoblast-like cells. While these cell populations did not mineralize, alkaline phosphatase activity increased in response to $1,25(OH)_2D_3$ and decreased in response to PTH and osteocalcin production increased in response to $1,25(OH)_2D_3$. The cAMP response to PTH was variable. Following transformation, cell population 13-1 displayed osteoblast-like characteristics similar to those expressed prior to transformation. Furthermore, cell population 13-1 expressed these same characteristics when place at the non-permissive temperature of 37^{0}C. Unlike cell population 13-1, cell population 16-3 displayed a phenotype more characteristic of fibroblast-like cells. Both 13-1 and 16-3 have been

passaged over 30 times and may be considered immortal. Thus, these cell populations can be maintained in continuous culture over many generations, but may be studied under conditions when cellular metabolism resembles primary cells by placing them at the non-permissive temperature. By contrast, the transforming factors can not be turned off in cell population 14-4, which underwent spontaneous transformation by an unknown mechanism.

SUMMARY

Alternative culture methods did not result in selection of cell populations from the periodontal ligament enriched in the osteoblast-like phenotype. Regardless of the culture media, cell populations were found to display characteristics representative of the phenotypic heterogeneity inherent in the periodontal ligament. While four cell populations which mineralized were suggestive of a more mature, differentiated osteoblast-like phenotype, most cell populations expressed *in vitro* markers consistent with a less differentiated osteoblast-like phenotype. Transformation of periodontal ligament cells was possible using transfection with the SV-40 large T antigen and the temperature sensitive construct allowed the transformation gene to be turned off at the higher, non-permissive temperature. Three immortalized periodontal ligament cell populations have been established and may be useful for future *in vitro* study into the functional and regulatory properties of this tissue.

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Vita

Michael Edward Poth was born in New Haven, Connecticut on September 5, 1963 to Dr. James Edward and Alice Faye Poth. Following graduation from Talawanda High School in Oxford, Ohio in May, 1981, he attended Miami University in Oxford, Ohio where he obtained a Bachelor of Arts degree in Zoology. After graduating from Miami University in May 1985, he was admitted into The Ohio State University school of dentistry in Columbus, Ohio in August that same year. During dental school, he was elected to membership in the Omicron Kappa Upsilon National Honor Society. Graduating Cum Laude, he received the degree of Doctor of Dental Surgery in June, 1989. He was commissioned as an officer in the United States Air Force and entered service in July, 1989, where he began a one year General Practice Residency at Wright-Patterson Air Force Base in Dayton, Ohio. Upon completion of his residency in July, 1990, he was assigned as a staff general dentist to Aviano Air Base, Italy. He started graduate training in periodontics at Wilford Hall Medical Center, Lackland Air Force Base, Texas and The University of Texas Health Science Center at San Antonio in June, 1995. During his residency, he was honored for his work in research by being selected as a finalist in the 1997 American Association for Dental Research Edward H. Hatton Award. He was admitted for candidacy for the Master of Science degree at the University of Texas Graduate School of Biomedical Sciences at San Antonio in January of 1998. He was married on April 25, 1992 to Karen S. Andreassi and they have one son, Justin Edward, born on May 9, 1996.